A transcriptomics approach to study the molecular mechanisms regulating interleukin-10 gene expression in T helper cells

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Declaration

I Charlotte Frances Whicher confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Interleukin-10 (IL-10) is an immunoregulatory cytokine that has a vital role in maintaining a balanced and appropriate immune response. CD4+ T helper (Th) cells are important in regulating an effective immune response, and are a dominant source of IL-10. Despite the different signalling pathways that result in the polarisation of each Th subset and lead to the expression of their hallmark cytokines, IL-10 is expressed by all of the different Th subsets. We show that Th1 cells cultured with IL-12 produce IFNγ and small amounts of IL-10, while Th1 cells cultured with IL-12 and IL-27 produce large amounts of IL-10 and IFNγ. Furthermore, we show that Th17 cells can produce IL-10, or not, depending on the presence of IL-2. However, these Th cell populations are phenotypically heterogeneous, particularly with respect production of IL-10 protein. Less than half of each of these *in vitro* cultured Th cell populations expresses IL-10 or the hallmark cytokine, and co-expression of IL-10 and the hallmark cytokine is also heterogeneous. Therefore we devised and implemented an innovative new technique that enables RNA-Seq analysis of different intracellular cytokine producing cell subpopulations from within Th subsets. We find that it is possible to extract high quality mRNA from Th samples, even though they have been fixed and stained for intracellular cytokines, and that the data from these samples is replicable. Using this technique we have identified potential molecules and pathways by which (I) IL-27 drives IFNγ and IL-10 production by Th1 cells, and (II) IL-2 drives IL-10 production by Th17 cells. Furthermore, by separating different intracellular cytokine producing subpopulations within different Th1 and Th17 cell subsets we have found that cytokine producing and non-cytokine producing subpopulations within heterogeneous Th subsets have significantly different transcriptional profiles and that some pathways and molecules are enriched in IL-10 producing cells.

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Table of Contents

Table of Figures

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

List of Tables

Chapter 2

Chapter 5

Chapter 6

Abbreviations

The International System of Units (SI) was used for abbreviations of units. Standard notations were used for chemical elements and formulae. Gene and protein names follow standard abbreviations as described on ZFIN and are also clarified in the text. Other abbreviations used in this work are listed below:

AhR	Aryl hydrocarbon receptor
AKT-mTOR	Akt-mammalian target of rapamycin
AP-1	Activator protein 1
APC	Antigen presenting cell
ARE	Adenosine-uridine-rich elements
ATF	Activating transcription factor
AUF	ARE/poly(U) binding degradation factor
BCR	B Cell receptor
BfA	Brefeldin A
BM	Bone marrow
CD	Cluster of differentiation
cDNA	Complemntary DNA
ChIP	Chromatin immunoprecipitation
CMV	Cytomegalovirus
CNS	conserved non-coding sequences or central nervous system
CpG	Cytosine-phosphate-guanine
DASL	cDNA-mediated Annealing, Selection, extension, and Ligation
DC	Dendritic cell
DLL	Delta-like Notch ligands
EAE	Experimental autoimmune encephalitis
EBV	Epstein-Barr virus
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence assisted cell sorting
FFPE	Formalin-fixed paraffin-embedded
GC	Glucocorticoids
GFP	Green fluorescent protein
GO	Gene ontology
GR	Glucocorticoid receptor

GRE	Glucocorticoid response element
HSS	Hypersensitive sites
IBD	Inflammatory bowel disease
ICOS	Inducible costimulator
ICS	Intracellular cytokine staining
IFN	Interferon
IL	Interleukin
Iono	Ionomycin
IPA	Ingenuity Pathway analysis
IRF	Interferon regulatory factor
ISH	*in situ* hybridisation Janus kinase
JAK	Janus kinase
JDP	JUN dimerising protein
KLF	Kruppel-like factor
LCMV	Lymphocytic choriomeningitis virus
LPS	Lipopolysaccharide
mDC	Myeloid dendritic cells
MHC	Major histocompatibility complex
miRNA	Micro Ribonucleic acid
MOG	myelin oligodendrocyte glycoprotein
mRNA	Messenger Ribonucleic acid
MS	Multiple sclerosis
MyD88	Myeloid differentiation primary-response protein 88
NF-kB	Nuclear factor-kB
NFAT	Nuclear factor of activated T cells
NFIL	Nuclear factor of interleukin
NIP	NFAT interacting protein
PCA	Principle component analysis
PCR	Polymerase chain reaction
PDBu	Phorbol 12,13-dibutyrate
pDC	Plasmacytoid dendritic cells
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
PRR	Pattern recognition receptors

RA	Retinoic acid
RI	Ribonuclease inhibitor
RIN	RNA integrity number
SAP	SLAM-associated protein
SLAM	Signalling lymphocytic activation molecule
SLE	Systemic lupus erythematosus
SMAD	Signalling lymphocytic activation molecule
SNP	Single nucleotide polymorphisms
SOCS	Suppressor of cytokine signalling
STAT	signal transducer and activator of transcription
TCR	T cell receptor
Tfh	T follicular helper cell
TGF	Transforming growth factor
Th	T helper
TIR	Toll/IL-1 receptor
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TPL	Tumour progression locus
TRIF	TIR-domain-containing adaptor protein inducing IFNb
TSS	Transcription start site
TTP	Tristetraprolin
TyK	Tyrosine kinase
UTR	Un-translated region
VitD3	1,25(OH)2-vitamin D3
WT	Wild type
YFP	Yellow fluorescent protein

Chapter 1. Introduction

1.1 A overview of the immune response

The immune system has evolved many mechanisms with which to respond to the constant assault of potentially pathogenic organisms and promote normal health of the host. In jawed vertebrates these mechanisms involve cells that can be broadly categorised into the innate and adaptive immune systems, which act in a co-ordinated way to protect the host against infection. The innate immune system is thought to be evolutionarily older than the adaptive and is the first line of defence against invading pathogens. It controls the initial infection, activates the adaptive immune response and dictates the course the following immune response will take. However, unlike the adaptive immune system, the innate has restricted specificity and cannot prevent re-infection, as it has limited immunological memory (Sun et al., 2009). The adaptive immune system is more evolutionarily advanced, and it can recognise pathogens specifically and mount robust memory responses. The different receptor types these cells use to recognise pathogens dictate the difference in specificity of these two branches of the immune system. Innate immune cells express germ line encoded, invariant receptors that have broad specificities for pathogens; while adaptive immune cells express receptors encoded by various genes which are recombined resulting in a diverse repertoire of receptors (Medzhitov, 2007).

The innate immune system is comprised of many different cell types including dendritic cells (DCs), macrophages, natural killer cells, basophils, eosinophils and mast cells. DCs and macrophages recognise infection via ligation of the germ-line encoded pattern recognition receptors (PRRs), expressed on their surface, by conserved protein motifs expressed by microorganisms. When infection is detected by the innate immune system an inflammatory response is initiated, resulting in the recruitment of other immune cells to the site of infection and the production of the soluble factors chemokines and cytokines (Medzhitov, 2007).

DCs and macrophages are also known as antigen presenting cells (APCs) as they alert cells of the adaptive immune system to infection by migrating to the lymphoid organs of the host and stimulating T cells. APCs take up pathogens, process them and present the peptide antigen on their surface in the context of major histocompatibility complexes (MHC) to T cells. These will ligate the T cell receptor (TCR) and alongside ligation of

co-stimulatory molecules, the T cells will become activated (Guermonprez et al., 2002); they will clonally expand and differentiate into effector cells. There are two main types of effector T cells; the CD8+ cytotoxic T cells recognise antigen presented on MHC class I and directly kill infected cells (Harty et al., 2000), therefore they mainly recognise intracellular pathogens; and the CD4+ helper T cells that recognise antigen presented on MHC class II, and can activate other cells of the immune response including innate cell types and B cells (Abbas et al., 1996). Innate cells produce cytokines, which alongside the activation of CD4+ T cells by the ligation of the TCR, influence the expansion of CD4+ T cells and direct their differentiation into distinct subsets of T helper (Th) cell that are specialized to eradicate different pathogens through differing effector functions (**Figure 1.1**) (Murphy et al., 2000).

Another arm of the adaptive immune response is formed by the B cells, which produce antibodies and have many crucial immunological functions. Like T cells, B cells can be separated into many lineages, which have various roles in the immune response. The terminally differentiated effector lineage of plasma B cells are activated by ligation of the B cell receptor (BCR) by antigen and respond by secreting pathogen specific antibodies to neutralise pathogens (Kurosaki et al., 2010). However, other B cell subsets exist that can express multiple cytokines and present antigen, which is internalised upon BCR ligation, and expressed in the context of MHC class II (Mauri and Bosma, 2012). After the elimination of an antigen most immune cells undergo programmed cell death, however a few remain to form the pool of memory T and B cells. These ensure a faster and more effective immune response upon secondary invasion by the pathogen, and therefore provide long lasting immune protection.

Alongside the eradication of pathogens, however, is the risk of over-exuberant immune responses that can cause more damage to the host than the invading pathogen. Therefore parallel mechanisms within the immune response are in place to limit host damage and prevent reactivity to self. One important way in which the immune system does this is via the soluble factor Interleukin-10 (IL-10). This cytokine is crucial in maintaining a balanced immune response (Jankovic et al., 2010), it is produced by multiple cell types and has immunoregulatory functions on a broad range of targets (**Figure 1.2**) (Moore et al., 2001; Saraiva and O'Garra, 2010).

1.2 The role of T helper cells in the immune response

For over fifty years we have known that the thymus is crucial in immune system development; neonatal thymectomy in mammals leads to impaired immune responses (Kay, 1970). In fact the thymus is the environment in which thymus (T) cells develop from haematopoietic stem cells. The thymus has four discrete areas, which have different microenvironments with specific functions that guide T cells through the stages of development. The T cells can be traced through their development by alterations in the expression of markers on their cell surface; these include the TCR, CD3, CD4, CD8, CD25 and CD44. The expression of CD4 and CD8 enables the separation of T cells into the early stage double negative (CD4-CD8-), intermediate stage double positive (CD4+CD8+) and final stage single positive (CD4+ or CD8+) cells. The differential expression of CD25 and CD44 allows the separation of the four main double negative sub-stages (Koch and Radtke, 2011). Within the thymus the genes encoding the TCR are randomly rearranged and the T cells undergo a process of selection where cells with TCRs that have a high affinity for self peptide-MHC complexes are eliminated by apoptosis and those with potentially useful TCR specificities are allowed to mature (Mingueneau et al., 2013; Yamane and Paul, 2012). This entire developmental process results in the removal of self-reactive cells and the existence of a diverse pool of defensive T cells.

In the 1960s the first functional roles were discovered for T cells. A series of papers were published showing that thymus-derived cells were necessary for antibody production, but that these cells themselves were not making the antibodies. In fact they were somehow supporting the production of antibodies from bone marrow (B) -derived cells, and therefore these cells acquired the name T helper cells (Reviewed in (Crotty, 2015). Twenty years later, in 1986 two distinct CD4+ Th clones were identified, IFNγ producing Th1 cells and IL-4 producing Th2 cells (Mosmann et al., 1986). Since then different Th cells, such as Th17, Treg and T follicular helper (Tfh) cells, have been reported based on their transcription factor expression and cytokine secretion profile.

1.2.1 The differentiation of functionally heterogeneous T helper cell subsets

The differentiation of Th subsets from the homogenous naïve CD4+ T cell pool requires three signals. The first involves the presentation of peptide antigen on MHC class II molecules by APCs to the TCR of the T cell. The second involves the interaction of ligands on the APCs surface, such as CD80/86, with TCR costimulatory molecules found on the surface of T cells, such as CD28 (Yamane and Paul, 2013). The third signal involves the presence of polarising cytokines within the microenvironment of the Th cell; signals from these cytokines lead to the expression of signal transducer and activator of transcription protein (STATs) and transcription factors that direct the effector function of that cell (O'Garra, 1998). The culmination of these three signals leads to the activation and initiation of differentiation of the Th cell into a specific Th cell subset that can coordinate the eradication of the invading pathogen (**Figure 1.1**).

1.2.1.1 An overview of the T helper cell subsets

Each T helper subset has a specific differentiation profile; polarising cytokines instruct the cell along a certain differentiation pathway; STATs and transcription factors are activated in accordance with the polarising cytokines and act on genes within the T cell to dictate which cytokines are produced upon infection. The different Th subsets have been rigorously studied and they are now characterised by the 'hallmark' cytokines that they produce, and their 'master' transcription factors that dictate their differentiation. Different Th subsets have different phenotypic characteristics as they participate in different types of immune and inflammatory responses. The profiles of these subsets are outlined in **Figure 1.3**.

A hallmark cytokine is considered to be a cytokine that is produced by a specific subset of Th cell that drives a specific immune response. IFNγ is the hallmark cytokine of Th1 cells that acts on macrophages leading to their accumulation at the site of infection and the clearance of intracellular pathogens (O'Garra, 1998). IL-4, alongside IL-5 and IL-13, are the hallmark cytokines of Th2 cells (Sher and Coffman, 1992), which act to clear helminths by activating mast cells (IL-4) and eosinophils (IL-5), and elevating class switching and IgE production by B cells (IL-4 & IL-13) (Zhu, 2010). IL-17 is the hallmark cytokine of Th17 cells (Harrington et al., 2005), which is involved in the

defence of mucosal barriers (Ciofani et al., 2012; Korn et al., 2009). Alongside these hallmark cytokines these cells also produce other cytokines. In particular, all Th subsets produce IL-10, alongside other pro-inflammatory cytokines, which acts to feedback negatively on the immune response (Jankovic et al., 2010; Saraiva and O'Garra, 2010). A master transcription factor is defined by its expression being necessary and sufficient for the differentiation and acquisition of specific characteristics of a certain cell type (Josefowicz, 2013). T-bet is considered the master transcription factor for Th1 cells (Szabo et al., 2000), GATA3 for Th2 cells (Zheng and Flavell, 1997), RORγt for Th17 cells (Ivanov et al., 2006), FoxP3 for Treg cells (Josefowicz et al., 2012) and BCL6 for Tfh cells (Johnston et al., 2009); however recent evidence suggests that the role of these factors may be more limited than initially thought and they may need to act collaboratively with other factors (Josefowicz, 2013). This will be discussed in greater detail later in the chapter.

1.2.1.2 The early signalling events that underlie T helper cell differentiation

The affinity of ligation of the TCR and its costimulatory molecules, and the dose of antigen, determine the strength of stimulation of naive CD4+ T cells and is a major checkpoint in their differentiation. The strength of stimulation can also be regulated by serial triggering, where a few MHC-antigen complexes trigger multiple TCRs (Valitutti et al., 1995), and by the duration of TCR-MHC interactions (Iezzi et al., 1998). The required duration of TCR signalling for T cell activation varies between naïve and effector T cells; naïve T cells need as much as 20 hours of signalling to initiate proliferation, while effector T cells need only 1 hour for initiation of commitment with prolonged TCR stimulation of effector T cells resulting in cell death (Iezzi et al., 1998). This can be circumvented by CD28 costimulation, which facilitates greater signalling in reduced periods of TCR-MHC interaction (Iezzi et al., 1998). The strength of TCR signalling can regulate the Th1-Th2 and Th17-Treg cell balance *in vitro*. Strong signalling leads to Th1 differentiation (Nembrini et al., 2006) and weak favours Th2; strong TCR stimulation leads to high levels of extracellular signal-regulated kinase (ERK) activation that restricts the early production of GATA3 and the activation of STAT5 by IL-2 is blocked, therefore blocking the induction of Th2 cells and supporting Th1 development (Yamane and Paul, 2013). In the presence of TGFβ weak TCR

signalling leads to FoxP3 expression and failure of T cells to differentiate into Th17 cells (Gabrysova et al., 2011).

Antigen dose can similarly have a fundamental impact on the differentiation of Th cells. Low levels of antigen dose, as with weak TCR signalling, lead to the development of Th2 cells while high levels of antigen presented by DCs leads to the development of Th1 cells (Constant et al., 1995; Hosken et al., 1995). In mice high levels of TCR occupancy by large amounts of antigen lead to greater ERK phosphorylation and the downregulation of GATA3 (O'Garra et al., 2011). A similar effect has been seen in human cells; DCs cultured with LPS and pulsed with high levels of antigen drove Th1 cell differentiation, while those that were pulsed with low doses of antigen drove Th2 cell differentiation (Langenkamp et al., 2000). There is some evidence that at very high antigen doses Th2 cell differentiation is favoured again, the mechanism driving this is unknown, though it has been shown to be IL-4 dependent (Hosken et al., 1995). High concentrations of antigen have also been associated with Th17 cell differentiation; this is via a feedback mechanism where high antigenic stimulation leads to increased cell surface expression of CD40L which cross-talks with CD40 on DCs to increase their IL-6 production, which further promotes Th17 polarisation (Iezzi et al., 2009). Additionally it has been suggested for Th1 cells that abundant and repeated antigenic stimulation may result in IL-10 production from these cells, and reduced IL-2 production and anergy (Gabrysova et al., 2009; Saraiva et al., 2009). The signalling strength from the TCR controls downstream expression of cytokine receptors (van Panhuys et al., 2014), which is the next step in determining the polarisation of naïve CD4+ T cells.

The dominant signals that orchestrate naïve CD4+ T cell specification are cytokines. These are soluble proteins released by various cell types in response to infection, that initiate and direct the immune response. Cytokines in the microenvironment surrounding the naïve CD4+ T cells bind receptors on their surface and promote or repress certain signalling cascades that drive different differentiation pathways. IL-12 is a cytokine produced predominantly by macrophages and DCs upon encounter with microbial products, and is the main factor in directing Th1 cell differentiation and IFNγ production (Hsieh et al., 1993b). Ligation of the IL-12 receptor leads to a signalling cascade that activates STAT4 and drives Th1 cell development; mice where the IL-12 gene or STAT4 gene has been deleted (knockout mice) have markedly reduced levels of

Th1 responses (O'Garra, 1998). IL-4 is the crucial cytokine in the initiation of Th2 cell development (Le Gros et al., 1990; Seder et al., 1992; Swain et al., 1990). Ligation of the IL-4 receptor leads to a signalling cascade that activates STAT6 and drives Th2 cell development (Hou et al., 1994). The source of this IL-4 remains elusive, with many cell types being suggested, but no consensus having been reached; many cell types produce IL-4 but the relevance of this in the context of driving Th2 cells is unknown and is probably context depenedent. Other than autocrine T cell derived IL-4, which is known to propitiate Th2 cell differentiation (Abbas et al., 1996), basophils have centrally been discussed as having a role in driving Th2 cells, with studies advocating their promotion of Th2 cell development (Sokol et al., 2008; Sullivan et al., 2011). The cytokines required to drive Th17 cell differentiation are numerous and thought to act in a certain order; with TGFβ, activating SMADs, and IL-6, activating STAT3, to induce initial differentiation, IL-21 subsequently driving amplification via STAT3 and finally IL-23 maintaining stability via STAT3 and STAT4 (Yamane and Paul, 2013). Alongside the 'driving' cytokines, IL-2 plays a fundamental role in the differentiation of all the Th subsets. IL-2 signals through the IL-2 receptor and activates STAT5. IL-2 acts on all known Th subsets, but in different ways. It has been shown to be essential for Th1 and Th2 differentiation, and to block Th17 and Tfh differentiation, via different mechanisms (Yamane and Paul, 2012).

1.2.1.3 The role of pioneer transcription factors and STATs in T helper cell differentiation

The differentiation of T helper cells can be considered as a two-step process; the first step being the ligation of the TCR and initial activation of the cells, and the second being the distinction of the Th subset phenotype by the cytokines present in the microenvironment. The initial TCR signals seem to result in pioneer transcription factors initiating an initial phase of gene regulation by altering the enhancer landscape of the cell's chromatin (Vahedi et al., 2013b). In Th17 cells BATF and IRF4 are thought to act as pioneer factors by cooperating to control chromatin accessibility and enable subsequent RORγt binding and the binding of other transcription factors that are activated by cytokine stimulation (Ciofani et al., 2012; Glasmacher et al., 2012; Li et al., 2012a). BATF is part of the activator protein 1 (AP-1) family of proteins, other members of which have been shown to have a role in pre-patterning of chromatin in

other cell types (Biddie et al., 2011). IRF4 has been shown to contribute to the development of multiple Th subsets and therefore may collaborate with other AP-1 family members to act as a pioneer in different Th subsets (Ahyi et al., 2009; Li et al., 2014), though this is still unknown. Pioneer factors in Th1 and Th2 cells remain elusive, this is likely to be due to the fact that unlike RORyt, T-bet and GATA3 have direct effects on the epigenetic landscapes of cells (Vahedi et al., 2013a). In fact GATA3 is expressed at varying levels throughout T cell development, and GATA3 binding can precede enhancer activation during thymocyte development. This suggests that alongside being a master transcription factor for Th2 cells, GATA3 may also be a pioneer factor (Wei et al., 2011; Zhang et al., 2012).

Most specifying cytokines exert their actions through STATs, which can alter the enhancer landscape of the chromatin and bind directly to genes as transcription factors. Unlike master transcription factors, whose activity is regulated by the level of expression, STATs are mostly regulated by cytokine-mediated post-translational modifications (Zhu et al., 2010). STATs not only induce and collaborate with master transcription factors to drive cytokine expression, but they also play an important role in the induction of other transcription factors (Zhu et al., 2010). STATs play a crucial role in altering the chromatin landscape; STAT3, STAT4 and STAT6 have all been shown to recruit and regulate the binding of the acetyltransferase p300 to specific genes (Ciofani et al., 2012; Vahedi et al., 2012), and in the absence of these STATs the master transcription factors cannot recover the enhancer landscapes of the cells (Wei et al., 2010). However, cytokines often activate more than one STAT family member and multiple cytokines usually act on a cell; therefore the role of STAT signalling within differentiating Th cells is multifaceted, where they can act in complex or opposition with one another (O'Shea et al., 2011). Recently the negative regulatory role of STATs has become more apparent; for instance STAT4 can inhibit Th2 differentiation while promoting Th1, and STAT6 can inhibit Th1 differentiation while promoting Th2 (Zhu et al., 2010). Interestingly, the *Il10* gene is positively regulated by both STAT4 and STAT6 (Saraiva and O'Garra, 2010). STATs can also act competitively, such as in Th17 cells where STAT3 and STAT5 compete for multiple common binding sites, and where it is a balance of the two signals that determines the lineage specification of the cell rather than the magnitude of each signal (Yang et al., 2011).

1.2.2 The role of different T helper cell subsets in the immune response

1.2.2.1 T helper 1 cells (Th1)

Invasion by intracellular pathogens causes DCs and macrophages to produce IL-12 (Hsieh et al., 1993b), which activates STAT4 in naïve T cells (O'Shea et al., 2011) and, via IFNγ, induces the expression of the transcription factor T-bet (Lighvani et al., 2001) and the reprograming of chromatin structure to activate the *Ifng* gene (Szabo et al., 2000). IFNγ is the hallmark cytokine of Th1 cells and acts on macrophages, leading to their accumulation at the site of infection and the clearance of intracellular pathogens, such as *Mycobacterium tuberculosis* (O'Garra, 1998). However alongside this protective role of Th1 cells can be dysregulation, leading to immunopathology and autoimmune or inflammatory diseases (Gabrysova et al., 2009).

1.2.2.1.1 The role of Th1 cells in the immune response

It has been known for many years that Th1 cells are predominantly induced by bacteria, such as during *B. abortus* (Scott and Kaufmann, 1991) and *M. tuberculosis* (Redford et al., 2011) infection. However, Th1 cells are also found in large numbers in the initial stages of malaria infection with Plasmodium parasite (Langhorne et al., 1989), and in other parasitic infections such as *Trichinella* and *Leishmania* (Scott and Kaufmann, 1991). Additionally, Th1 cells have been found to be induced during viral infections such as Hepititis C virus (Brady et al., 2003) and HIV (Clerici and Shearer, 1993). Th1 activation is often time dependent, as seen in Malaria, and location dependent, as seen in the compartmentalisation of Th responses to different organs in *Trichinella* infection (Scott and Kaufmann, 1991). Infection of the host by these pathogens will lead to IL-12 secretion by DCs and macrophages that will drive the differentiation of Th1 cells, as described above. These activated Th1 cells will then release IFNγ, which then drives specific immune responses to eradicate the invading pathogen. The IFNγ receptor is expressed by almost all cells within the body, however Th1 cell mediated IFNγ is mainly thought to act on DCs and macrophages. It leads to the enhanced expression of the MHC class II receptors on the surface of DCs and macrophages, leading to more effective antigen presentation to CD4+ T cells (Billiau et al., 1998). Furthermore, IFNγ increases the production of iNOS and other anti-microbial factors by macrophages that

aid in the killing of pathogens (Stuehr and Marletta, 1987). Additionally Th1 IFNγ inhibits Th2 cell development (O'Garra, 1998).

1.2.2.1.2 Transcriptional regulation of Th1 cell differentiation and cytokine production

IL-12 and IFNγ activate STAT4 and STAT1, respectively, which in turn drive the expression of *Tbx21*, the gene encoding the T-box protein T-bet (Zhu et al., 2010). Studies have shown that though IFNγ activates STAT1 during Th1 cells differentiation, it is not crucial for Th1 development and functioning. Nevertheless, it is thought that when the Th1 response is sub-optimally stimulated, the activation of STAT1 becomes more important (Zhu et al., 2010). The important role IFNγ signalling plays in Th1 responses is highlighted by findings that mutations in humans in the IFNγ receptor and STAT1 lead to susceptibility to mycobacterial disease (Fortin et al., 2007). Unlike STAT1, STAT4 has been shown to have a vital role in Th1 development, with STAT4 deficiencies leading to a discrete phenotype of decreased IFNγ production. STAT4 has been shown to have an important role in epigenetically modifying the chromatin landscape of cells and a core set of genes that are highly dependent on direct STAT4 binding have been described, including *Ifng* and *Tbx21* (Wei et al., 2010). STAT4 has also been found to actively repress STAT6 target genes in Th1 cells, therefore repressing Th2 lineage genes (O'Shea et al., 2011).

T-bet is considered to be the master regulator of Th1 cells (Szabo et al., 2000). It drives the Th1 differentiation programme by initiating IFNγ and IL-12 receptor expression, creating a positive feedback loop. T-bet also maintains the Th1 lineage and antagonises the differentiation of Th2 and Th17 cells by inhibiting GATA3 and RORγt (Li et al., 2014). However, T-bet is not alone in driving the Th1 lineage, it acts in complex with other transcription factors; including runt-related transcription factor 3 (RUNX3), enabling the induction of *Ifng* and repressing the expression of *Il4* (Djuretic et al., 2007). T-bet can also cooperate with BCL6 to form repressive complexes that silence certain genes to promote Th1 development (Oestreich et al., 2011). In fact it has been shown that a deficiency in Blimp-1, a transcription factor that negatively regulates BCL6, leads to increased Th1 cell numbers (Lin et al., 2014); supporting the role for BCL6 in Th1 differentiation.

1.2.2.2 T helper 2 cells (Th2)

Parasitic invasion will result in the release of the polarising cytokine IL-4 which acts on naïve CD4+ T cells (Maggi et al., 1992; Swain et al., 1990) to activate STAT6 (Hou et al., 1994), inducing the expression of the transcription factor GATA3 (Scheinman and Avni, 2009; Zhu et al., 2001), which goes on to reprogram the chromatin structure of the cell activating the genes for *Il4*, *Il5* and *Il13* (Lee et al., 2000; Maier et al., 2012). These interleukins are the Th2 hallmark cytokines, which act to clear helminths by activating mast cells and eosinophils, and elevating class switching and IgE production by B cells (Zhu, 2010). The adverse side of this response is when it is mounted against otherwise innocuous environmental 'allergens', resulting in atopy and allergy (Akdis et al., 2004).

1.2.2.2.1 The role of Th2 cells in the immune response

Extracellular parasites, particularly gastrointestinal helminths, are the main stimuli of Th2 cell responses (Finkelman et al., 1991; Locksley, 1994; Scott and Kaufmann, 1991; Sher and Coffman, 1992). Helminth parasites such as *H. polygyrus* and *N. brasiliensis* are often used as mouse models of parasitic worm infection and are strong drivers of the Type 2 response (Mowen and Glimcher, 2004). Ligation of the TCR in combination with IL-4 leads to STAT6 signalling and initiation of the Th2 phenotype. However alongside IL-4, IL-2 signalling has been shown to be important in Th2 cell differentiation; IL-2 regulates IL-4 expression alongside promoting cell growth and survival (Ansel et al., 2006; Le Gros et al., 1990). After the initial stimulation of Th2 cells, the cells enter a state of rest during which they do not actively express Th2 cytokines. However, the chromatin is remodelled and Th2 specific genes become transcriptionally accessible. This enables further stimulation to drive Th2 cell cytokine secretion (Zeng, 2013). Activated Th2 cells release the cytokines IL-4, IL-5 and IL-13, which have specific and overlapping roles in driving Type 2 immunity. The genes for these cytokines are all clustered together on the same chromosome (chromosome 11 in mouse, and chromosome 5 in human) in an area known as the Th2 cytokine locus. The Th2 cytokines, IL-4, IL-5 and IL-13, act on epithelial cells, goblet cells and mast cells to drive mastocytosis, mucus production and eosinophilia, and on B cells to drive IgE production and MHC class II upregulation. IL-4 aids in B cell class switching of IgG

and IgE production, IL-4 and IL-13 affect macrophage activation, and IL-5 recruits eosinophils (Zhu, 2010). IL-4 is fundamental to the Th2 immune response; *in vivo* studies reveal that IL-4 knockout mice have impaired, but not absent, responses to helminth infection (Kopf et al., 1993), and IL-4 can compensate for the triple knockout of IL-5, IL-9 and IL-13 (Fallon et al., 2002). The activation of the Type 2 response leads to immunological actions that are pathogen specific, for instance expulsion of *N. brasiliensis* requires increased mucus production while expulsion of *T. spiralis* requires mast cell activation (Mowen and Glimcher, 2004). Th2 cells also make substantial amounts of IL-10 alongside their hallmark cytokines; explaining why IL-10 was initially described as a Th2 specific cytokine (Fiorentino et al., 1989).

1.2.2.2.2 Transcriptional regulation of Th2 cell differentiation and cytokine production

An initial round of low-antigen dose TCR stimulation leads to the upregualtion of GATA3 and IL-2 expression (Yamane et al., 2005), this IL-2 feeds back onto the cells and activates STAT5, which alongside GATA3 alters the accessibility of the IL-4 locus (Zhu et al., 2003). Paracrine and autocrine IL-4 act on the T cells to drive the differentiation of Th2 cells by activating STAT6. STAT6 is thought to be a crucial switch signal in the initiation of Th2 differentiation, and it has been shown to facilitate the expression of over half of the IL-4 regulated genes (O'Shea et al., 2011). STAT6 acts on both promoters of the *Gata3* gene to drive its expression; redundantly on the distal promoter and non-redundantly on the proximal promoter (Scheinman and Avni, 2009). TCR signalling leads to activation of nuclear factor of activated T cells 1 (NFAT1), which cooperates with STAT6 at the *Gata3* promoters to drive GATA3 expression (Scheinman and Avni, 2009). The IL-4/STAT6 signalling pathway also induces growth factor independent 1 (GFI-1) (Zhu et al., 2002), which promotes the proliferation of GATA3 high cells and suppresses the differentiation of other Th subsets (Zhu et al., 2006). Independently of the STAT6 signalling pathway, DCs and macrophages express Jagged proteins on their surfaces that ligate the Notch 1 and 2 proteins on CD4+ T cells and may induce Th2 cell differentiation by enhancing GATA3 transcription (Zeng, 2013).

GATA3 is considered to be the Th2 master regulator, however it is present at various levels through haematopoiesis and is expressed at low levels in naïve CD4+ T cells

(Wei et al., 2011). However, upon Th2 cell polarisation its levels are dramatically upregulated and it goes on to further activate its own expression (Lee et al., 2000; Ouyang et al., 2000). GATA3 over-expression can drive Th2 cell differentiation even under Th1 polarising conditions (Zheng and Flavell, 1997) or in STAT6 deficient cells (Ouyang et al., 2000), and down-regulation of GATA3 impairs the expression of all Th2 cell associated cytokines (Zheng and Flavell, 1997). GATA3 has been shown to be able to remodel chromatin and it directly binds to the promoters of *Il5* and *Il13* (Kishikawa et al., 2001; Lee et al., 1998); which are both highly responsive to GATA3. The *Il4* promoter, however, is less responsive to GATA3 (Lee et al., 2001). GATA3 has also been shown to act on various enhancers of the Th2 cytokine locus (Zeng, 2013). GATA3 also induces the transcription factor c-Maf, which cooperates with JUNB to drive expression of the *Il4* gene (Li et al., 1999a), and synergises with Kruppel-like factor 13 (KLF13) at the *Il4* promoter to regulate IL-4 expression (Kwon et al., 2014). Finally, alongside promoting various aspects of Th2 differentiation, GATA3 can suppress Th1 differentiation by attenuating the IL-12/STAT4 signalling axis (Ferber et al., 1999; Ouyang et al., 1998; Usui et al., 2003; Zeng, 2013).

Independently of the IL-4/STAT6 signalling pathway, TCR signalling leads to the activation of NFAT, AP-1 and nuclear factor-κB (NF-κB); all of which contribute to Th2 cytokine gene activation. NFAT and AP-1 together bind the NFAT/AP-1 composite site at the *Il4* promoter to activate it (Rooney et al., 1995), while NF-κB synergises with NFAT1 and nuclear factor of IL-6 (NFIL6) to activate the *Il4* promoter (Li-Weber et al., 2004). There is also overlap between the TCR signalling and IL-4/STAT6 signalling pathways, mostly through interactions with c-Maf. Interferon regulatory factor 4 (IRF4) physically interacts with NFATc2 to activate the *Il4* promoter, and these together synergise with c-Maf to drive IL-4 expression (Rengarajan et al., 2002). Furthermore, NOD-like receptor family, pyrin domain containing 3 (NLRP3), the expression of which in Th2 cells is regulated by STAT5 via IL-2 signalling, directly interacts with IRF4 to facilitate optimal IRF4-dependent *Il4* expression (Bruchard et al., 2015). The NFAT interacting protein NIP45 has also been shown to interact with NFATc2 and c-Maf to activate the *Il4* promoter (Hodge et al., 1996).

1.2.2.3 T helper 17 cells (Th17)

Bacterial or fungal invasion will lead to IL-6 and TGFβ acting on naïve CD4+ T cells to drive the activation of STAT3 and SMADs, respectively, (Bettelli et al., 2006; Veldhoen et al., 2006; Yamane and Paul, 2013) that induce the transcription factor RORγt which in turn drives the hallmark cytokine IL-17 and IL-22 expression (Awasthi and Kuchroo, 2009). However, IL-1, IL-21 and IL-23 have all also been implicated in playing important accessory roles in Th17 differentiation, and Th17 cells can also produce IL-6, IL-21 (Korn et al., 2009), and IL-10 (McGeachy et al., 2007), depending on the stimulatory conditions. Initially most of the effector functions of Th17 cells were described in the context of autoimmunity, however more recent data suggests a vital role for Th17 cells in immunity, particularly in the defence of mucosal barriers (Ciofani et al., 2012; Korn et al., 2009; Puel et al., 2010).

1.2.2.3.1 The role of Th17 cells in the immune response

Th17 cells were initially established as a separate lineage of Th cells in the context of the autoimmune disease EAE, where Th17 cells specific for autoantigens induce severe tissue inflammation (Awasthi and Kuchroo, 2009; Langrish et al., 2005). Nevertheless, the evolutionary function of Th17 cells is not to cause autoimmunity and therefore there must be situations in which the massive inflammation driven by Th17 cells is necessary. There is now a wealth of evidence to show that Th17 cells are involved in mounting immune responses to bacterial and fungal infections, and they are thought to be particularly important in the defence of mucosal barriers (Aujla et al., 2007). Neutralisation of the IL-23/IL-17 axis prevents mice for clearing *Pneumocystis* infection, highlighting their important role in anti-fungal responses (Rudner et al., 2007). Mice deficient in IL-17 and IL-23 are also susceptible to gram-negative bacteria such as *K. pneumonia*, and IL-17 and IL-22 have been shown to increase proliferation of lung epithelial cells and resistance to injury by this bacterium (Aujla et al., 2008). IL-17 has also been shown to have important anti-microbial functions, for example during infection with *M. tuberculosis* (*Mtb*); Th17 cells have been implicated in the early response to *Mtb* and are thought to be crucial in recruiting Th1 cells to the site of infection (Awasthi and Kuchroo, 2009; Khader et al., 2007).

The cytokines that Th17 cells release have different wide-spread roles in controlling and recruiting cells to maintain immune responses to bacterial and fungal infection. Overall Th17 cells are implicated in upregulating certain chemokines, pro-inflammatory cytokines and colony-stimulating factors (CSFs) to induce neutrophils and recruit myeloid cells to the site of infection. As mentioned above the IL-23/IL-17 axis is important in many Th17 cell actions; IL-23 acts to feedback on Th17 cells to drive greater IL-17 expression and has been suggested to repress the expression of immunoregulatory cytokines such as IL-10 (McGeachy et al., 2007). Furthermore, IL-23 driven Th17 cells have a greater ability to express IFNγ and behave in a pathogenic manner (Ghoreschi et al., 2010; Langrish et al., 2005; Lee et al., 2009b). IL-17, alongside IL-22, acts on many different cell types and has a broad range of proinflammatory effects. IL-21, however, has more targeted actions on certain immune cells, such as B cells to drive their expansion and isotype class-switching, and on Th17 cell themselves to initiate further amplification of the Th17 response (Korn et al., 2009). However, the actions of IL-21 are complicated by findings that it has immunosuppressive roles; it is thought to regulate immune responses by inducing IL-10 production by CD4+ and CD8+ T cells (Spolski et al., 2009).

1.2.2.3.2 Transcriptional regulation of Th17 cell differentiation and cytokine production

IL-6, IL-21 and IL-23 all signal through STAT3 to drive Th17 differentiation. Deletion of STAT3 in CD4+ T cells leads to detrimental effects on IL-17 and IL-21 expression, and STAT3 has been shown to directly bind the *Il17* and *Il21* genes. Additionally, STAT3 has been shown to bind multiple genes implicated in Th17 differentiation, including *Rorc* which encodes the Th17 master transcription factor RORγt, alongside genes for the transcription factors BATF and c-Maf, and the receptors for IL-23 and IL-6 (O'Shea et al., 2011).

Unlike GATA3 and T-bet, RORγt alone is not sufficient to drive Th17 cell differentiation. Alongside STAT3, which has been shown to be critical for IL-17 and IL-21 expression, other transcription factors have been shown to regulate IL-17 and RORγt, including BATF and IRF4. In fact an absence in BATF leads to defective Th17 polarisation (Li et al., 2014). Recent ChIP-Seq studies have revealed that it is actually

likely that a large complex of transcription factors co-localise at the promoters of *Il17* and *Il21* to act competitively or cooperatively to drive gene expression. The transcription factors suggested to be involved thus far include STAT3, RORγt, BATF, IRF4, c-Maf, JUN/JUNB/JUND and FOSL2 (Ciofani et al., 2012; Li et al., 2012a).

Both paracrine and autocrine TGFβ are thought to be important in Th17 cell differentiation, though the role of TGFβ in driving Th17 differentiation is complex for two main reasons. Firstly the concentration of TGFβ in the environment is crucial in dictating Th cell differentiation; low doses of TGFβ can synergise with IL-6 and IL-21 to initiate the development of Th17 responses, however high doses of TGFβ lead to the inhibition of IL-23 receptor expression and the induction of regulatory T cells expressing the transcription factor FoxP3 (Awasthi and Kuchroo, 2009). Secondly, it has been shown that Th17 cells can be polarised in the absence of TGFβ; if the cells are driven with IL-1β together with STAT3 activation then the IL-23 receptor can be upregulated, and this IL-23 responsiveness enables the generation of pathogenic Th17 cells in the absence of TGFβ (Ghoreschi et al., 2010). Nevertheless, TGFβ is generally considered to be required for Th17 induction. Unlike other Th17 driving cytokines, TGFβ signals through the SMAD family of proteins; activation of the TGFβRI leads to phosphorylation of SMAD2 and 3 which complex with SMAD4 to translocate to the nucleus and transcribe genes. Deletion of SMAD2 prevents Th17 cell differentiation, and studies suggest that SMAD2 may control the cross-talk between TGFβ and IL-6 (Malhotra et al., 2010). While the deletion of SMAD3 leads to an increase in Th17 differentiation, the double knockout of SMAD2/3 leads to reduced Th17 differentiation but no effect on RORγt; suggesting the upregulation of RORγt by TGFβ is not via SMAD2/3 (Takimoto et al., 2010). Recent studies have found that though TGFβ signals through both SMAD2 and SMAD3, these proteins actually have differential effects on STAT3 driven transcription of IL-17. In support of previous findings, SMAD2 promotes STAT3 transactivation of *Rorc* and *Il17a* while SMAD3 promotes inhibition of STAT3 (Yoon et al., 2015). TGFβ has also been shown to interact with NFAT and IRF4 to drive the transcription of IL-23 (Hermann-Kleiter and Baier, 2010). SOCS3 is activated by IL-6 and IL-21 signalling, but feeds back as a negative regulator of STAT3 signalling, and the deletion of this factor leads to increased Th17 numbers; TGFβ inhibits SOCS3 to prolong STAT3 activation in Th17 cells (Qin et al., 2009). Additionally, TGFβ may also aid Th17 differentiation by inhibiting the differentiation

of both Th1 and Th2 cells (Gorelik et al., 2002; Gorelik et al., 2000; Zhu and Paul, 2010).

Finally, IL-1β has also been shown to promote the proliferation and maintenance of antigenically stimulated Th17 cells in the context of some inflammatory conditions. In particular it has been shown that in the absence of TGFβ, IL-1β is crucial in aiding IL-6 and IL-23 driven differentiation of Th17 cells (Ghoreschi et al., 2010; Zielinski et al., 2012). It acts through the Akt-mammalian target of rapamycin (Akt-mTor) pathway via glycogen synthase 3 (GSK3) and IRF4 to regulate IL-21 expression (Muranski and Restifo, 2013).

1.2.3 T helper cell lineage commitment

1.2.3.1 Heterogeneity of cytokine production within T helper cell populations

Initial studies of Th1 and Th2 cell populations established that the cells making up these subsets express cytokines in a heterogeneous manner (Bucy et al., 1994; Murphy et al., 1996; Openshaw et al., 1995). By looking at single cells within each subset using double labelling of cytokines with *in situ* hybridisation (ISH) it was ascertained that certain cytokines are more or less likely to be coexpressed. In IL-4 driven Th2 cells, IL-4 and IL-5 are highly coexpressed at all stages of stimulation, while IL-4 and IL-10 are increasingly coexpresssed with multiple rounds of cell stimulation (Bucy et al., 1995). It is now well established that heterogeneity exists within all Th cell subsets, and this can be visualised by their patterns of cytokine production; as each lineage is able to express multiple cytokines. This heterogeneity in cytokine secretion is likely to be due to differences in epigenetic modifications, temporal and stochastic signalling events and the variable expression of transcription factors (O'Garra et al., 2011; Zhu et al., 2010).

However, the origins and factors controlling the heterogeneity within Th cell subset populations remain unclear. The heterogeneity in Th2 cells has in part been associated with the ETS family transcription factor PU.1, which has been shown to prevent GATA3 binding the *Il4* locus resulting in lower levels of IL-4 expression. When PU.1 expression is attenuated the homogeneity of the Th2 population is increased (Chang et al., 2005). In Th17 cells it has been shown that activation of the aryl hydrocarbon

receptor (AhR) may dictate the expression of IL-17 and IL-22; in the absence of the AhR IL-22 expression by Th17 cells is lost while IL-17 expression is only mildly affected (Veldhoen et al., 2008a). Nevertheless establishing the factors that underlie Th subset cytokine expression heterogeneity remains a problem. However, new technologies, including single cell RNA-Sequencing, are being applied to finds answers to questions such as these; a topic that will be discussed in greater detail later in this chapter.

1.2.3.2 Feedback and cross-regulation between T helper cell subsets

Each Th subset has a set of molecular feedback mechanisms instilled within it to maintain the differentiation of that specific lineage. In most Th subsets, the master transcription factor, often in concert with other transcription factors, drives increased expression of cytokine receptors that leads to positive feedback loops; promoting cell fate determination (Zhu et al., 2010). As already mentioned, in Th1 cells IFNγ activation of STAT1 drives T-bet expression, which then initiates further IFNγ production (Lighvani et al., 2001). Additionally, in human T cells T-bet feeds back to directly activate its own expression and re-enforce the Th1 lineage (Kanhere et al., 2012). With regard to Th2 cells, the hallmark cytokine IL-4, which is expressed by Th2 cells, is also the critical driving cytokine of Th2 cells, and therefore the actions of IL-4 on Th2 cells drives further IL-4 expression and reinforcement of this lineage. Dec2 is a transcription factor that has been shown to directly bind *Junb* and *Gata3* and drives their expression, and GATA3 has been shown to regulate Dec2 expression, creating a positive feedback circuit that drives expression of GATA3 and enhancement of Th2 differentiation (Yang et al., 2009). Additionally Dec2 has been shown to upregulate IL-2 receptor expression, which could further boost Th2 responses (Liu et al., 2009). In Th17 cells RORγt induces the expression of IL-21, which then acts on the Th17 cells to initiate further STAT3 signalling and maintenance of RORγt expression (Murphy and Stockinger, 2010). However, alongside these positive feedback mechanisms that further drive Th subset differentiation, there are circumstances in which Th cells will feedback negatively on themselves or each other to reduce differentiation and proliferation. One of the main mechanisms by which Th cells do this is via the production of the immunosuppressive factor IL-10 (O'Garra and Vieira, 2007).

While maintaining certain Th lineages, cytokines can also suppress the differentiation of other lineages. IL-4 suppresses Th1, Th17 and Treg differentiation via the upregulation of GFI-1, and IL-2 suppresses the Th17 lineage while promoting Th1, Th2 and Treg cells in appropriate contexts. TGFβ promotes Th17 and Treg development and prevents Th1 and Th2 cell differentiation (Gorelik et al., 2002; Gorelik et al., 2000; Zhu and Paul, 2010). Transcription factors also play a crucial role in cross-regulation of Th cell subset differentiation at both transcriptional and post-transcriptional levels. For instance, T-bet inhibits GATA3 and RORγt function and therefore antagonises the differentiation of Th2 and Th17 cells. One of the mechanisms by which it does this is by associating with RUNX1 and therefore preventing RUNX1 and RORγt cooperation in Th17 differentiation (Li et al., 2014). Additionally, T-bet in Th1 cells induces RUNX3, which directly binds *Il4* and represses its expression, and can repress GATA3 function (Zhu and Paul, 2010). However in Th2 cells, GATA3 can interact with RUNX3 to prevent its upregulation of IFNγ expression (Yagi et al., 2010) and GATA3 can downregulate STAT4 expression (Usui et al., 2003). At the post-transcriptional level, phosphorylated T-bet can interact with GATA3 to suppress its capacity to drive IL-5 expression (Hwang et al., 2005). Enforced STAT5 signalling in Th1 cells can drive Th2 differentiation in the absence of GATA3 upregulation (Zhu et al., 2003), and STAT5 has been shown to suppress RORγt and IL-17 upregulation (Laurence et al., 2007). Overexpression of RORγt can prevent STAT4 activation and T-bet upregulation (Mukasa et al., 2010), and it can bind *Foxp3* to repress its expression (Burgler et al., 2010). IL-6 can also suppress FoxP3 function in Tregs through STAT3, while conversely FoxP3 can interact with RORγt to prevent IL-17 expression (Zhou et al., 2008). The mechanisms by which cytokines and transcription factors repress certain genes expressed in different Th subsets to reinforce the differentiation of that subset are complex and diverse, but are fundamental in maintaining certain types immune responses.

1.2.3.3 T helper cell subset plasticity

Once a naïve CD4+ T cell has differentiated into a specific Th subset, can that effector phenotype be changed to better control infection? There are two mechanisms by which the phenotype of CD4+ T cells can be changed during infection: cellular plasticity and population plasticity. Cellular plasticity refers to individual cells changing their

phenotype and cytokine secretion profile, while population plasticity refers to changes in the size of different Th populations due to proliferation or cell death (Magombedze et al., 2013). Population plasticity is a generally accepted phenomenon, and though all the processes behind driving the changes in Th cell numbers are not understood, it is clear that during infections, particularly chronic infections, the ratios of different Th subsets shift (Zhang et al., 1997). However, the concept of cellular plasticity is still an area of contention.

It has been shown *in vitro* (Murphy et al., 1996) and *in vivo,* with *N. brasiliensis* infection (Panzer et al., 2012), that Th1 cells can acquire the characteristics of Th2 cells. However, *in vitro* it has been shown that the more 'terminally' differentiated a cell becomes, after multiple rounds of antigenic stimulation, the harder it is to reprogram (Murphy et al., 1996). This model may be reflected *in vivo* during chronic infections, such as with the CRTh2^{+} pool of Th2 effector memory cells described by Messi et al (Messi et al., 2003) which have no flexibility and are not capable of reverting to the Th1 profile under any conditions tested. At least some plasticity can be attributed to Th2 cells differentiating from naïve T cells still present within the Th1 cell populations. However, even Th2 cells that are considered stably committed, that have lost the expression of the IL-12Rβ2, can be reprogrammed to re-express the IL-12Rβ2 and produce both IL-4 and IFNγ (Hegazy et al., 2010; Murphy and Stockinger, 2010) and some Th2 populations can express both GATA3 and T-bet (Zhu and Paul, 2010). The potential for plasticity in IFNγ expression may be due to the bivalent histone modifications of H3K4me3 and H3K27me3 found at the *Tbx21* locus (Wei et al., 2009). Bivalent modifications at master transcription factor gene loci may be one of the key factors in enabling Th cell subset plasticity. Another example of the reprogramming of a Th cells is in the presence of TGFβ Th2 cells can acquire the capacity to produce IL-9 (Veldhoen et al., 2008b), while IL-9 can also be expressed by Th17 cells. *In vivo* Th17 cells are commonly found to acquire the potential to express IFNγ (Bending et al., 2009; Hirota et al., 2011; Lee et al., 2009b), and in some cases they even lose the ability to express IL-17 (O'Shea and Paul, 2010). These examples argue for plasticity in Th cell cytokine expression and the blurring of lines between the different Th subsets, but the actual frequency of Th cells that alter their cytokine profiles at the single cell level *in vivo* remains uncertain, and with current technologies it is not possible to test.

1.3 The role of IL-10 in the immune response

IL-10 was initially named cytokine synthesis inhibitory factor when it was discovered in 1989 (Fiorentino et al., 1989) and it was described as a product of Th2 cells which acted on Th1 cells to suppress their differentiation and effector functions. The wider role of IL-10 was suggested 2 years later with the finding that IL-10 could inhibit many macrophage functions, including the release of proinflammatory cytokines, to indirectly reduce the actions of CD4+ T cells (Bogdan et al., 1991; Fiorentino et al., 1991a; Fiorentino et al., 1991b). IL-10 was also found to inhibit DC IL-12 production, preventing them from driving Interferon-γ (IFNγ) production by CD4+ and CD8+ T cells (Hsieh et al., 1993a; Macatonia et al., 1993). IL-10 is produced by most cells of the immune system (**Figure 1.2**) and is now considered to be a crucial immunoregulatory cytokine with various actions (Saraiva and O'Garra, 2010). IL-10 deletion in mice can lead to inflammatory bowel disease and other exaggerated inflammatory disorders, highlighting its importance in limiting inflammation (Moore et al., 2001). Though this phenotype is not seen in germ free mice, emphasising the importance of IL-10 in the balance between the immune system and commensal bacteria.

IL-10 is a homodimeric protein and part of the IL-10 family of cytokines that includes IL-9, IL-20, IL-22, IL-24, IL-26, IL-28A, IL-28B and IL-29 (Ouyang et al., 2011). These can be subgrouped based on their chromosomal location, structure, receptor usage and biological functions. However, this family has very diverse biological functions and only IL-10 has been clearly associated with an anti-inflammatory role (Ouyang et al., 2011). Additionally, in Epstein-Barr virus (EBV) and in human cytomegalovirus (CMV), viral homologues of the *Il10* gene have been described, these are now known as ebvIL-10 (Moore et al., 1990) and cmvIL-10 respectively (Kotenko et al., 2000). The existence of viral IL-10 suggests there is a beneficial role of mimicking the actions of IL-10 for viruses. This is supported by that fact that though ebvIL-10 mimics the immunosuppressive actions of IL-10 (Hsu et al., 1990), it does not result in the immunostimulatory actions (Vieira et al., 1991).

1.3.1 Cellular sources of IL-10

It has been established that IL-10 is produced by a range of cells in both the innate and adaptive immune systems (Maynard and Weaver, 2008; Moore et al., 2001). Within the innate system DCs, macrophages, mast cells, natural killer (NK) cells, eosinophils and neutrophils have all been shown to express IL-10 (O'Garra and Vieira, 2007). Recognition of microbe-derived products by pattern recognition receptors (PRRs) causes DCs and macrophages to become activated and express cytokines, including IL-10. IL-10 is produced rapidly by these cells upon toll-like receptor (TLR) and other PRR ligation and is therefore thought to be important in controlling early responses to pathogens and acute inflammation (Murray, 2006b). IL-10 production by mDCs and macrophages can be induced downstream of TLR2, TLR3, TLR4 and TLR9 (Kaiser et al., 2009; Saraiva and O'Garra, 2010). Furthermore, other PRRs can be potent inducers of IL-10, such as Dectin-1 in mDCs (Slack et al., 2007). The amount of IL-10 expression by DCs and macrophages does differ, with macrophages producing high levels of IL-10, myeloid DCs producing intermediate amounts and plasmacytoid DCs producing none (Boonstra et al., 2006). Independently of TLRs, ligation of Dectin-1 leads to potent induction of IL-10 in myeloid DCs but not macrophages (Reviewed in (Saraiva and O'Garra, 2010), suggesting that innate cells may not have the same intrinsic capacity to make IL-10.

Alongside their role as positive regulators of the immune response, B cells do also have suppressive roles in the immune response, chiefly through their production of IL-10. IL-10 production by B cells as part of their normal immune response was first shown in 1990 (O'Garra et al., 1990), and this was followed by a series of publications highlighting Ly-1+ CD5+ B-1 cells, which are the main producers of natural antibodies, as the major B cell source of IL-10 (O'Garra et al., 1992). Since then our understanding of B cell IL-10 expression has greatly improved (Fillatreau et al., 2002; Hilgenberg et al., 2014) and there is now a B cell subset specifically attributed to IL-10 production and immune system downregulation, known as Bregs (Mizoguchi et al., 2002). This initial paper found that in a spontaneous colitis model, CD1d hi CD5+ B cell derived IL-10 could suppress Th2 mediated immunity. So far no marker has been found to define Bregs, however various subtypes of Breg have now been proposed, with different phenotypes depending on environmental stimuli, which are thought to all stem from a common progenitor (Mauri and Bosma, 2012).

The concept of T cell mediated immunosuppression is an old one, and the mechanisms by which CD8+ T cells facilitate this are varied, though mostly they are considered to function via the cytolytic killing of other immune cells harbouring the pathogen. However, the expression of IL-10 and immune repression via this mechanism has not been greatly explored. Nevertheless, there is evidence that both human and mouse CD8+ T cells do express IL-10 and can downregulate the immune response. In mice, tolerant CD8+ T cells, which did not proliferate in response to PMA and ionomycin or IL-2, constitutively expressed IL-10 (Tanchot et al., 1998). Furthermore, in mice, MHC class I restricted CD8+ T cells can be activated to develop a regulatory phenotype that is mediated by contact-dependent suppression of other T cells in combination with IL-10 expression (Noble et al., 2006). In humans, monocyte-derived DCs can induce CD8+ T cell differentiation into non-cytolytic IL-10 producing T cells that can inhibit APC induced CD8+ T cell proliferation (Gilliet and Liu, 2002). IL-10 expressing CD8+ T cells are thought to be most common intestinal epithelial lymphocytes found in the small intestine of naïve mice (Maynard and Weaver, 2008).

Unlike CD8+ T cells, the production of IL-10 by CD4+ T cells has been extensively studied, as they are considered the major source of IL-10 in inflammation and infection; conditional deletion of IL-10 production in CD4+ T cells closely resembles the phenotype with *T. gondii* infection seen in non-conditional IL-10 deficient animals (Roers et al., 2004). As mentioned, IL-10 was initially thought to be a Th2 subset specific cytokine, produced alongside IL-4, IL-5 and IL-13 (Fiorentino et al., 1989). However, it is now known to be expressed by a wide variety of Th cells. This includes Th1 cells that express IL-10 alongside IFNγ; these cells often have a strong effector phenotype suggesting they produce IL-10 as a regulatory feedback mechanism to control their own actions (Jankovic et al., 2007; O'Garra and Vieira, 2007; Svetic et al., 1993; Trinchieri, 2001). Additionally, Th17 cells have been shown to conditionally express IL-10 (McGeachy et al., 2007; Stumhofer et al., 2007), and IL-9 producing Th cells (Th9 cells) also produce IL-10 (Veldhoen et al., 2008b). Regulatory CD4+ T cell (Treg) populations, distinguished by the expression of the transcription factor FoxP3, can either be derived in the thymus or induced from naive T cells in the periphery (Hori et al., 2003; Sakaguchi, 2005; Uhlig et al., 2006). These cells are regarded as an immunosuppressive CD4+ T cell subset that make IL-10 as part of their multiple suppressive mechanisms (Josefowicz et al., 2012). Tregs release IL-10, which can

promote their survival whilst downregulating other immune responses; maintaining an optimum balance between pathogen removal and host-mediated inflammatory pathology (Trinchieri, 2001). Additionally, it has been suggested that there is a population of FoxP3 negative Tregs which are derived in the periphery that only produce the cytokine IL-10, often referred to a Tr1 cells (Gregori et al., 2010; Maynard et al., 2007; Roncarolo and Gregori, 2008; Vieira et al., 2004); making them distinct from the other Th cell subsets such as Th1, Th2 and Th17, which secrete IL-10 and have regulatory roles, but also may secrete other effector cytokines.

1.3.2 Cellular targets of IL-10

The IL-10 receptor is expressed on most haematopoietic cells and is composed of two receptor chains, IL-10R1 and IL-10R2 that are members of the IFN receptor family (Moore et al., 2001). IL-10R1 is important for the binding of IL-10, while the accessory subunit IL-10R2 is critical for signal transduction. IL-10R2 is also used in signalling for other IL-10 family cytokines, highlighting that the IL-10R1 chain is the important portion for IL-10 specificity (Ouyang et al., 2011). DCs and macrophages express the highest levels of the IL-10R, supporting evidence that these are the main targets of IL-10 activity (Murray, 2006b).

Upon ligation of the IL-10 receptor Janus kinase (JAK) 1 and tyrosine kinase (TyK) 2 are activated, JAK1 in turn phosphorylates the IL-10R1 chain and TyK2 phosphorylates the IL-10R2 chain (Ouyang et al., 2011), enabling docking with the SH2 domains of signal transducer and activator of transcription (STAT) 3 (Finbloom and Winestock, 1995). The IL-10 receptor can also activate other STATs and initiate other signalling pathways, however STAT3 is thought to be the most important in the anti-inflammatory effects of IL-10 (Murray, 2006b). However, IL-10 is not the only receptor to signal via STAT3; IL-6 also signals via STAT3, but IL-6 cannot initiate the anti-inflammatory response that IL-10 does. It has been proposed that this may be due to temporal differences in the signalling pathways, with SOCS3 terminating IL-6 signalling before it can initiate the anti-inflammatory pathway (Murray, 2006a).

IL-10 is thought to signal via STAT3 to induce the expression of inhibitory factors, which in turn limit proinflammatory cytokine and chemokine production (Murray,

2005). The actions of these inhibitory factors are thought to act mainly via transcriptional inhibition, such as in human macrophages where IL-10 signalling leads to the inhibition of elongation of the tumour necrosis factor (TNF) transcript (Smallie et al., 2010). IL-10 may also have post-transcriptional actions; it has been shown to negatively regulate TNFα production by mediating the expression of microRNA-187 (Rossato et al., 2012). Additionally, IL-10 has been shown to inhibit IL-12p40 production by inhibiting *Il12b* transcription in myeloid cells and this has been proposed to be via the expression of the STAT3-dependent transcription factor nuclear factor of interleukin 3 (NFIL3) that mediates *Il12b* transcription inhibition (Smith et al., 2011). Further genome-wide studies have proposed other IL-10 signalling targets, the relevance of which are yet to be confirmed (Hutchins et al., 2012b).

In the 1990s it was suggested that IL-10 also has direct immunomodulatory effects on CD4+ T cells, reducing Th1 and Th2 proliferation and cytokine production (De Waal Malefyt et al., 1993). However these effects were variable and controversial, as it has been shown that IL-10R1 is downregulated on T cells upon activation (Moore et al., 2001), and may have been explained by the effects of contaminating DCs or macrophages in the culture. It is now generally accepted that IL-10 has no direct effect on Th1 cells, but instead limits the ability of DCs and macrophages to promote their differentiation and proliferation. It does this by downregulating the expression of the following DC and macrophage surface molecules: MHC, intracellular adhesion molecule-1 and the costimulatory molecules CD80 and 86 (Maynard and Weaver, 2008; Moore et al., 2001). Additionally, as discussed above, IL-10 reduces the transcription of pro-inflammatory cytokines from DCs and macrophages; such as IL-12, which drives Th1 cell differentiation (Moore et al., 2001). More recent studies, however, have shown that Th17 cells express the IL-10 receptor, and that when the activity of this receptor is specifically blocked on these cells there is an increase in Th17 cell numbers during intestinal inflammation (Huber et al., 2011). Additionally it has been shown that IL-10 acts directly on Treg cells; (I) to maintain their FoxP3 expression and hence suppressive functions (Murai et al., 2009); (II) to enhance their suppression of Th17 cells via STAT3 (Chaudhry et al., 2011); (III) to increase their own IL-10 production which goes on to act at IL-10 receptors on Th17 cells (Huber et al., 2011). IL-10, therefore, reduces the inflammatory actions of Th cells, in both an indirect and a direct manner.

Paradoxically, alongside the suppressive effects of IL-10 described above, IL-10 can also have certain stimulatory effects on the immune system; such as the enhancement of NK cell proliferation and IFNγ production (Moore et al., 2001) and the activation of mast cells (O'Garra et al., 2008). It can also enhance the activation of polyclonal B cells, which can have a protective role or a pathogenic role; such as in systemic lupus erythematosus (O'Garra et al., 2008). IL-10 has been shown to upregulate MHC class II expression on the surface of naïve mouse B cells, and to enhance their survival (Go et al., 1990). In humans IL-10 can also promote B cell survival by rescuing them from apoptosis, though this effect is dependent upon the activation state of the cells (Itoh and Hirohata, 1995). IL-10 has also been attributed to a role in B cell differentiation and isotype class switching (Moore et al., 2001). Originally a factor secreted by B cells was described to promote the proliferation of thymocytes in the presence of IL-2 and IL-4, this was found to be IL-10 (MacNeil et al., 1990), and further studies support the role of IL-10 in promoting the proliferation and cytotoxic activity of activated CD8+ T cells when in combination with low doses of IL-2 (Groux et al., 1998). As with B cells the effects of IL-10 on CD8+ T cells can be either enhancing or inhibitory, depending on the activation state of the cells.

1.3.3 Regulation of the immune response by IL-10

A proinflammatory immune response is critical for the protection against infection, but it can become pathogenic and cause damage to the host if it is over exuberant. Additionally inappropriate immune responses, such as against self antigens and non-harmful commensal or innocuous antigens, can also cause damage to the host. IL-10, with its immunosuppressive actions, plays a crucial role in preventing this excessive inflammation and autoimmunity. However, inappropriate IL-10 production when inflammation poses no threat to the host can prevent effective clearance of pathogens, and potential onset of chronic infections. Therefore the maintenance of a balanced immune response is crucial for normal functioning of the host, with the optimal amount of IL-10 ensuring protection from immunopathology but not inhibiting appropriate immune responses (Gabrysova et al., 2014; Moore et al., 2001).

1.3.3.1 The role of IL-10 in the protection against immunopathology

IL-10 knockout (KO) mice suffer from severe alterations in intestinal homeostasis and inflammatory bowel disorders such as chronic enterocolitis (Kuhn et al., 1993). However, germ-free mice do not develop this phenotype (Sellon et al., 1998), suggesting that this colitis formation is due to commensal bacteria in the gut activating the immune system; in IL-10 deficient mice there is no suppression of this immune response and therefore there is excessive immunopathology. There are multiple lymphocyte populations that regulate intestinal inflammation, however CD4+ T cell derived IL-10 is considered to be one of the key mediators of gut homeostasis (Roers et al., 2004). The important role for IL-10 in steady state intestinal immunity is reflected in humans, where coeliac disease and inflammatory bowel disease (IBD), which are the most common forms of non-infectious intestinal inflammation in humans, have been linked to single nucleotide polymorphisms (SNPs) flanking the *Il10* gene (Franke et al., 2008). Nevertheless, though IL-10 plays a non-redundant role in intestinal homeostasis, it is not critical to steady state systemic immune regulation. However when not in the steady state, such as in the context of infection, IL-10 plays a fundamental part in immunoregulation.

In the setting of infection, IL-10 is essential in limiting tissue damage resulting from unrestricted inflammation; it acts at different stages of the immune response and at various locations within the host. Endotoxic shock, which is characterised by an over-exuberant inflammatory response, multi-organ failure and possible death, can develop in response to lipopolysaccharide (LPS), which is found in the cell walls of gram-negative bacteria. The role of IL-10 in regulating this response is highlighted by the findings that disruption of the *Il10* gene in mice greatly reduced the amount of LPS required for a lethal dose, part of which was attributable to enhanced TNFα and IFNγ production (Berg et al., 1995). Subsequently, macrophages have been shown to be a crucial source of protective IL-10 in this model (Roers et al., 2004).

Much additional *in vivo* evidence supports the important role of IL-10 in immunity; IL-10 deficiency leads to lethal autoimmune responses as a reaction to certain parasite and bacterial infections such as *T. gondii* (Gazzinelli et al., 1996), *P. chabaudi chabaudi* (Li et al., 1999b) and *T. muris* (Schopf et al., 2002). In these infections a strong Th1 immune response, characterised by IFNγ production, is mounted in reaction to the

pathogen, and in the absence of IL-10 this response is not controlled and therefore there is enhanced mortality. In fact depletion of CD4+ T cells with anti-CD4 antibodies protects IL-10 deficient mice from mortality induced by *T. gondii* (Gazzinelli et al., 1996). This protection against excessive Th1 immune responses is also seen during murine cytomegalovirus infection, where lacking IL-10 leads to immunopathology related to excessive Th1 driven inflammation during infection (Oakley et al., 2008). Interestingly, in these infections where there is a highly polarised Th1 response, and therefore IL-10 producing Th2 cells are not present, the source of IL-10 seems to be the Th1 cells themselves (Jankovic et al., 2010; Jankovic et al., 2007). Therefore it is thought that these cells are in fact feeding back to regulate their proinflammatory immune responses and prevent immunopathology (O'Garra and Vieira, 2007).

Allergic diseases are complex conditions resulting from IL-4 driven Th2 cells mounting inappropriate inflammatory responses to innocuous antigens (Kay et al., 1991; Robinson et al., 1992; Till et al., 1997), such as pollen, which lead to high levels of IgE production and eosinophilia (Durham et al., 1992; Hawrylowicz and O'Garra, 2005; Kemeny et al., 1989). Recent evidence also suggests that IL-10 may play a protective role in allergy (Faith et al., 2009; Faith et al., 2012), and that these diseases may be caused by an imbalance between inflammatory Th2 cells and suppressive IL-10 producing Th cells (Ling et al., 2004). Healthy individuals have a shift towards IL-10 expressing Th cells, while allergic individuals have higher numbers of inflammatory allergen-specific Th2 cells (Akdis et al., 2004). Furthermore it has been demonstrated that Treg cells prevent allergen-specific Th2 cytokine responses in healthy (non-atopic) individuals (Cavani et al., 2003; Taams et al., 2002), in an IL-10 dependent manner (Xystrakis et al., 2006b). This supports the notion that allergic individuals may have impaired or altered Treg function (Hawrylowicz, 2005). The beneficial role of IL-10 in allergy is also supported by the findings that glucocorticoids, that are the main treatment for asthma, induce IL-10 synthesis by human T cells (Barrat et al., 2002; O'Garra and Barrat, 2003; O'Garra et al., 2008). Furthermore, the addition of 1,25(OH)2-vitamin D3 (VitD3) alongside glucocorticoids enhances human Th cell IL-10 synthesis and restores responsiveness to glucocorticoids in steroid resistant individuals, enhancing therapeutic responses (Xystrakis et al., 2006b). The role of glucocorticoids and VitD3 in regulating IL-10 will be discussed in greater detail later in this Chapter.

Based on the broad anti-inflammatory effects of IL-10 there have been multiple studies in animals to see if the administration of IL-10 could be beneficial in the context of inflammation. In the model of endotoxic shock, the administration of IL-10 protects mice from the LPS induced shock, at least in part by decreasing TNFα production (Howard et al., 1993), though this has not been reproduced in humans. IL-10 treatment has also been shown to be beneficial in experimental autoimmune encephalitis (EAE), though in other models it has been shown to exacerbate the disease (Asadullah et al., 2003). In colitis IL-10 treatment was successful in ameliorating the disease, but only if administered prior to disease onset; it could not reverse established inflammation (Herfarth and Scholmerich, 2002). These results have led to an interest in the potential clinical applications of IL-10. A variety of clinical trials on healthy volunteers have been undertaken with the aim of treating immune mediated inflammatory diseases with recombinant IL-10. Though initial results were encouraging, the ensuing larger blinded trials showed little therapeutic benefit with multiple side effects (O'Garra et al., 2008). This highlights again the complex role IL-10 plays in the immune response and that it has both suppressive and stimulatory actions.

1.3.3.2 The role of IL-10 in the inhibition of protective immune responses

Contrary to the beneficial role of IL-10 in preventing over exuberant proinflammatory responses, in some circumstances the anti-inflammatory actions of IL-10 can hinder protective immune responses. Inappropriate amounts of IL-10 can enable certain pathogens to persist in the host as it suppresses the immune responses required to clear them. For example, during *M. tuberculosis* infection, where mice deficient in IL-10 mount elevated Th1 responses and expel the pathogen more effectively, resulting in lower bacterial loads than wild type mice (Redford et al., 2011). Similarly, infection with the protozoan *L. major* can lead to long-term persistence of the pathogen in the host that is not seen in IL-10 deficient mice (Belkaid et al., 2002). These mice can completely clear the infection, though this loss in IL-10 activity is also associated with the loss in immunity to reinfection (Belkaid et al., 2002); reinforcing the idea that the immune system needs equilibrium between effector and regulatory mechanisms. Further to this, high levels of IL-10 have been correlated with sever visceral leischmaniasis in humans; Th cells that are FoxP3 negative have been found as the main source of IL-10 in this instance (Nylen et al., 2007). In the context of viral infection IL-10 can also

inhibit protective immunity; persistent infection with lymphocytic choriomeningitis virus (LCMV) leads to increased IL-10 production and impaired T cell responses in mice (Brooks et al., 2006), whereas mice lacking the IL-10 gene or IL-10 receptor blockade results in robust effector responses and clearance of the virus (Brooks et al., 2006; Ejrnaes et al., 2006). Recent data suggests that Type I IFNs may drive IL-10 production from DCs during LCMV infection, which results in suppressed antiviral T cell responses, and that interfering with type I IFN signalling can restore protective immunity to LMCV (Wilson et al., 2013). Similarly, negative regulation of Type I IFN signalling protects against intracellular bacterial infections including *Mtb* and *L. monocytogenes* (McNab et al., 2014; McNab et al., 2013).

Not only is the generation of a robust immune response desirable in infection, it is also necessary for effective vaccination regimes, and therefore theoretically IL-10 could have a detrimental effect on vaccination efficacy (O'Garra et al., 2008). In fact the neutralisation of IL-10 using antibodies, in the presence of soluble antigen leads to more effective priming of Th1 responses and these cells mount an enhanced response upon rechallenge (Castro et al., 2000). Further to this, a recent study has shown that if IL-10 is blocked at the time of vaccination with BCG, then the vaccination efficacy is enhanced and there is a reduction in bacterial load after rechallenge with *M. tuberculosis* (Pitt et al., 2012).

1.3.3.3 The role of IL-10 in autoimmune disease

Damaging immune responses to self antigens can occur in the absence of foreign antigens, such as in autoimmunity. Multiple sclerosis (MS) is a disease characterised by central nervous system (CNS) inflammation and is associated with Th1 and Th17 cell driven inflammation at the myelin sheath that surrounds nerve fibres and EAE is a mouse model of MS (Zamvil and Steinman, 1990). The role of IL-10 in EAE has been suggested to be protective, as IL-10 deficient mice develop an accelerated, more severe form of EAE and recovery is also impaired as compared to wild type mice (Bettelli et al., 1998). However treatment with IL-10 has led to contradictory results, depending on the timing and level of administration (O'Garra et al., 2008). Rheumatoid arthritis (RA) is a chronic inflammatory disease characterised by inflammation affecting the joints. TNFα has been shown to be a pathological factor in RA, as the treatment of RA

patient's cells with anti-TNFα antibodies leads to reduced proinflammatory cytokine production (Brennan et al., 1989) and TNFα inhibitors are now used clinically as an RA therapy (Feldmann and Maini, 2003). IL-10 has been suggested to be protective in this condition, as the neutralisation of IL-10 leads to increased proinflammatory cytokine levels (Katsikis et al., 1994). Conversely, a number of studies have looked at the serum and synovial fluid of RA patients and found a positive correlation between RA and IL-10 (Asadullah et al., 2003). These two diseases highlight the dual role of IL-10 in autoimmunity and the difficulty with targeting it therapeutically. IL-10 is associated with increased B cell activity and autoantibody production, and has stimulatory effects on B cell activation, differentiation and proliferation. In systemic lupus erythematosus (SLE), an autoimmune disease associated with autoantibodies recognising nuclear proteins, it has been suggested that IL-10 has an important role in pathogenesis of the disease (Iyer and Cheng, 2012). In fact IL-10 levels in SLE patients have been found to be higher than those in healthy controls and high levels of IL-10 correlated with clinical score (Park et al., 1998), and polymorphisms in the *Il10* gene have been associated with SLE development (Reviewed in (Beebe et al., 2002). Furthermore, the treatment of SLE blood mononuclear cells with anti-IL-10 antibodies leads to a reduction in the production of autoantibodies (Llorente et al., 1995).

1.3.3.4 The role of IL-10 in cancer

Multiple studies have looked at IL-10 in anti-tumour immune responses and its role is thought to be highly contextual. Firstly, STAT3, the molecule through which IL-10 signals, has been associated with tumour growth and function as it has been found to be highly expressed in many solid tumours and when constitutively activated STAT3 can have oncogenic effects (Murray, 2006b). On the other hand IL-10 has been shown to facilitate antitumor effects via stimulating cytotoxic CD8+ T cells in the tumour microenvironment, though contrastingly IL-10 conditioned DCs are thought to anergise CD8+ T cells towards melanoma antigens (Mocellin et al., 2005). There are many examples in which IL-10 has immunosuppressive effects in cancer models, however there are also many other studies suggesting it has an immunostimulatory effect (O'Garra et al., 2008). These contradictory effects are likely to be due to a combination of the complexity of actions IL-10 exerts on the immune response and the heterogeneous nature of cancer as a disease.

The vast array of immunological systems and diseases IL-10 has been found to play a role in verifies its importance in regulating the immune response.

1.4 Transcriptional regulation of genes

DNA is negatively charged and therefore the ensuing electrostatic repulsion prevents strands of DNA interacting closely enough to fit into the nucleus. To solve this problem DNA is coiled around highly basic histone proteins, to form chromatin. The fundamental component of eukaryotic chromatin are the nucleosomes, these are comprised of eight histone proteins with approximately 147 bases of DNA wrapped around them. These nucleosomes are then further compacted into fibres (Jiang and Pugh, 2009). The position of the nucleosome within the DNA and chemical modifications of the histones is key to gene regulation. The wrapping of DNA around the nucleosomes controls the access of transcription factors and enzymes to the genetic code, leading to a regulated process of gene expression. Additionally polypeptide chains from the histones, known as 'tails', extrude from the nucleosome core and can undergo a wide range of covalent modifications, such as acetylation or methylation. These post-translational histone modifications can act as markers indicating the regulatory state of the chromatin (Winter and Amit, 2014) and can be associated with different functional elements (Lenhard et al., 2012). Chromatin state varies across different cell types and can be altered by environmental conditions. The digestion of nuclear DNA with DNaseI allows for mapping of the chromatin landscape; it creates a distinctive map of cleavage alluding to the nucleosome depleted regions, which are indicative of accessible chromatin where transcription factors can interact with the DNA (Winter and Amit, 2014). Chromatin immunoprecipitation (ChIP) can be used to profile histone modifications; using specific antibodies, regions enriched with the histone modification of interest can be sequenced (Barski et al., 2007).

The spacing of nucleosomes, the presence of histone modifications and the alterations of DNA methylation are known as 'epigenetic marks' (Rothenberg, 2013). These make up the chromatin landscape and dictate the transcription of genes by regulating the binding of transcription factors. The first layer to the chromatin landscape is the accessibility of the chromatin. For transcription factors to bind, chromatin needs to be in

an ‘open’ state; the chromatin is unwrapped and not closely associated with nucleosomes, and the regulatory sites, which are revealed as DNase hypersensitive sites (HSS), are exposed (Winter and Amit, 2014). The second layer to the chromatin landscape is the histone modifications; the type and location of these modifications are indicative of different regulatory states. The presence of multiple modifications, and the ratio between them, can be used to predict a wide variety of different functional elements. For example, acetylation, as a general rule, is associated with activation. H3K4me is usually associated with the transcriptional start sites (TSS) of transcribed genes (Zhou et al., 2011) and active genes (Santos-Rosa et al., 2002). On the other hand H3K27me3 is usually associated with inactive chromatin regions and repressed genes (Zhou et al., 2011). Further complexity is added by the presence of multiple histone marks at one site (Wang et al., 2008), such as the bivalent modification of both H3K4me3 and H3K4me27 suggesting that the gene is in a poised state, waiting for either activation or repression as the cell differentiates (Bernstein et al., 2006). The final component of the landscape is DNA methylation at the cytosine residues of CpG (cytosine-phosphate-guanine) dinucleotides, which is associated with gene repression. Evidence suggests an intimate link between histone modifications and DNA methylation; with histone modifications establishing the basic DNA methylation profile in cells and DNA methylation feeding back to maintain histone modifications throughout cell division (Cedar and Bergman, 2009).

Each gene is encoded by an area of DNA, known as the gene locus, which is transcribed by RNA polymerase II resulting in the production of mRNA. Genes consist of introns, which are removed from the RNA by splicing, and exons, which remain present in mature RNA. Transcription is initiated at the TSS, which is immediately downstream of the promoter, and contains a large portion of regulatory information for transcriptional initiation of that gene (Lenhard et al., 2012). Most promoters are associated with high GC base content and are known as CpG islands, and unlike most CpG dinucleotides, CpG islands are not methylated (Cedar and Bergman, 2009). The 3’ un-translated region (UTR), which is found immediately after the translated region of the gene, also often contains regulatory information (Lenhard et al., 2012). DNaseI HSS are areas via which gene expression can be regulated, and are often associated with particular genes. Furthermore, distal regulatory elements, such as enhancers, can be connected with genes; these are areas that regulate the transcription of a gene regardless of their location relative to the promoter (Bulger and Groudine, 2011).

Cells are directed to become different cell types by the complex regulation of gene transcription, resulting in each cell type having a unique transcriptional programme. This programme is dependent on the binding of transcription factor proteins to specific 'consensus' DNA regions that regulate the mRNA transcription of a gene by RNA polymerase II. Gain or loss of function studies highlight that transcription factors are crucial in the determination of cellular phenotype, and that mutations in transcription factors can lead to disease (Villar et al., 2014). These transcription factors fine-tune the transcriptional programme of a cell in a spatial and temporal manner (Magnani et al., 2011). However, at a given time each transcription factor only occupies a small portion of its consensus regions (Zaret and Carroll, 2011). Therefore, there must also be factors in place that dictate the binding of transcription factors to their target sequences. These factors include the presence and distribution of epigenetic modifications, and the accessibility of promoters and gene transcription enhancers (Bulger and Groudine, 2011; Magnani et al., 2011). However, not only do epigenetic modifications and enhancers regulate transcription factor binding, but transcription factors can regulate the fate of a cell by remodeling the chromatin and establishing a new set of enhancers. Some transcription factors can alter nucleosome position and recruit histone modifying enzymes, without leading to any transcriptional effects on gene expression; these factors are known as pioneer transcription factors (Rothenberg, 2013; Samstein et al., 2012). These factors refine the chromatin landscape and direct the binding of future transcription factors and therefore dictate the transcriptional programme of the cell (Zaret and Carroll, 2011).

Although the chromatin landscape can regulate the binding of transcription factors, once bound the transcription factors can also feed back to change the properties of the surrounding chromatin. As mentioned, pioneer factors can recruit histone modifying enzymes that can prevent or promote the binding of further transcription factors. Additionally, the binding of a transcription factor can be key in the recruitment of other transcription factors to that site; transcription factors often bind regions of DNA in combination with other transcription factors (Villar et al., 2014). Conversely, transcription factors can act competitively by binding specific regions of DNA and blocking the binding of other factors that would have initiated transcription, resulting in the repression of gene expression (Yang et al., 2011). Therefore, transcription factors not only enhance gene transcription, but can also repress it.

1.5 The molecular mechanisms of IL-10 regulation

It is crucial for the immune response to maintain the balance between the inflammatory responses required to eradicate a pathogen and preventing excessive inflammation, which could harm the host. Many publications have highlighted the importance of the regulatory cytokine IL-10 in this balance and have published findings into the mechanisms by which its production is regulated in immune cells (Saraiva and O'Garra, 2010). However, the molecular mechanisms, transcription factors and epigenetic modifications involved in IL-10 regulation are still incompletely understood, and though many transcription factors have been reported to enhance IL-10 production in Th cells, the molecular mechanisms regulating *Il10* transcription in different immune cells, and the existence of common mechanisms between all IL-10 producing cells, remains unclear (O'Garra and Vieira, 2007; Saraiva and O'Garra, 2010).

1.5.1 The structure of the *Il10* gene locus

The gene encoding the IL-10 protein is found on chromosome 1 in both humans and mice, and it is found within the *Il10* gene family cluster (Kim et al., 1992). Upstream of *Il10* this cluster contains the genes for *Il19, Il20* and *Il24,* which are all members of the IL-10 gene family of cytokines. The *Il10* gene has a well-defined TSS and consists of 5 exons and 4 introns followed by a 3' UTR. Within the core promoter are TATA and CCAT (A or G in mice) elements, which are characteristic of a focused promoter that has been associated with transcription in an environmentally responsive, tissue specific manner (Lenhard et al., 2012). The *Il10* gene is highly conserved between humans and mice with many homologous conserved non-coding sequences (CNS), suggesting that these regions have high levels of functional relevance and are therefore evolutionarily constrained. Many recent studies have highlighted some of these CNS as enhancers of *Il10* gene expression (Ahyi et al., 2009; Jones and Flavell, 2005; Li et al., 2012a; Wang et al., 2005).

Important for the regulation of *Il10* gene expression is the capacity to change the structure of the gene locus. In macrophages, IL-10 can be expressed almost immediately upon stimulation with certain stimuli; this is reflected in the structure of the *Il10* locus. The HSS in the *Il10* gene of macrophages are somewhat sensitive to DNaseI digestion

even in the resting state. When stimulated with the PRR ligands LPS, CpG or zymosan A, five HSS were found in bone marrow (BM)-derived macrophages and one in BM-derived DCs (Saraiva et al., 2005). Unlike macrophages, in T helper cells the *Il10* locus requires large amounts of remodelling during differentiation before it becomes active. In naïve T cells only one HSS is found while differentiated Th1 and Th2 cells have many more HSS (Im et al., 2004; Jones and Flavell, 2005; Saraiva et al., 2005; Wang et al., 2005). Many, but not all, of these HSS are common between Th cells and macrophages.

The accessibility of the *Il10* locus has also been studied in the context of histone modifications. In macrophages that express IL-10, hyperacetlyation of histone H4, which is considered to be an active mark, can be detected at two HSS (Saraiva et al., 2005). In Th1 and Th2 cells the *Il10* locus has been shown to be bivalently marked with H3K4me3 and H3K27me3, which are active and repressive markers, respectively (Wei et al., 2010). The factors involved in altering the structure of the *Il10* gene locus and regulating these epigenetic modifications will be discussed in detail in this chapter.

1.5.2 Regulation of IL-10 production in T helper cells

T helper cells are vital in controlling and maintaining an appropriate immune response, balancing between inflammatory responses required to eradicate a pathogen and the regulation of excessive inflammatory responses which would harm the host. Interestingly, despite the different signalling pathways that result in the polarisation of each Th subset and to the expression of their hallmark cytokines, IL-10 is expressed by all the different Th subsets. However, in each subset different factors may be associated with the expression of IL-10, and therefore it is likely that there are both common and dissimilar processes involved in the regulation of IL-10 expression in Th cells. In fact IL-10 expression in each subset is thought to be tightly linked to the differentiation of that unique subset, for instance in Th1 cells STAT4 is needed of IL-10 expression, while in Th2 cells STAT6 is needed and in Th17 cells STAT3 is required (Saraiva and O'Garra, 2010). As IL-10 regulation seems to be highly intertwined with the differentiation of each Th subset, the model of hallmark cytokine gene regulation; where TCR ligation and STAT activation leads to pioneer factors altering the epigenetic landscape, allowing for the expression of lineage specific factors, is likely to apply to

IL-10 gene regulation as well (Gabrysova et al., 2014). The specific signalling cascades and transcription factor complexes that regulate IL-10 gene expression in each Th subset are still being established.

1.5.2.1 External factors that regulate IL-10 production in T helper cells

IL-10 expression is initiated in Th cells via multiple external factors, which result in signalling cascades of proteins and transcription factors. The two main ways by which IL-10 expression is instigated is via ligation of the TCR and cytokine receptors, which consequently leads to complex signalling pathways that drive IL-10 transcription; these will be discussed in greater detail later in this chapter. With regard to the TCR, the type of antigen and the strength of the stimuli can both regulate IL-10 production by Th cells. For instance in Th1 cells, it has been shown that when naïve T cells are cultured in the presence of IL-12 and low levels of antigen the cells produce IFNγ but not IL-10. However when the cells are cultured with IL-12 and increasing doses of antigen, the number of IL-10 expressing cells increases with the antigen dose while IFNγ expression is not affected. Therefore Th1 cells require IL-12 in conjunction with high levels of TCR stimulation to express IL-10 (Saraiva et al., 2009). It is possible that high antigen dose causes the Th1 cells to feedback to the APC to induce factors, such as Type I IFN and IL-27, which then go on to enhance Th1 cell production of IL-10 together with IFNγ. Indeed, other extracellular factors produced by APCs, alongside antigen and cytokines, have also been associated with IL-10 expression.

Direct cell-cell interactions have been associated with the induction of IL-10 in Th cells. In humans it has been shown that immature myeloid DCs (mDCs) (Jonuleit et al., 2000) and mature plasmacytoid DCs (pDCs) (Moseman et al., 2004) can drive the differentiation of IL-10 producing regulatory CD4+ T cells. pDCs express high levels of inducible costimulator ligand (ICOS-L) on their cell surface, and it has now been identified that a direct interaction of pDCs with naïve T cells via ICOS-L ligation of ICOS on the T cells drives the differentiation of IL-10 producing Treg cells (Ito et al., 2007). Furthermore, it has been suggested that IFNγ may promote the expression of ICOS-L on accessory cells, which in turn acts on the Th1 cells to drive IL-10 expression (Shaw et al., 2006). The interaction of Notch ligands, expressed on APCs, with Notch on CD4+ T cells has also been shown to impact Th cell differentiation. With regard to

IL-10 production, Delta-like Notch ligands (DLL) 1 and 4 have been shown to induce IL-10 in Th1 cells without much effect on IFNγ production (Kassner et al., 2010; Rutz et al., 2008). Recently, liver sinusoidal endothelial cells (LSECs), which express all 4 Notch ligands; DLL1 and 4, and Jagged 1 and 2, have also been shown to induce IL-10 expression in Th1 cells (Neumann et al., 2015). Additionally, the Notch ligand Jagged1, which has been associated with IL-4 production and Th2 differentiation, has been linked to driving IL-10 expression in IFNγ secreting human T cells (Kemper et al., 2003).

Immunosuppressive drugs, including Dexamethasone and 1,25(OH)2-vitamin D3 (VitD3), have also been implicated in regulating inflammatory cytokine genes and enabling the development of IL-10 expressing Th cells (O'Garra and Barrat, 2003; Xystrakis et al., 2006a). Glucocorticoids (GCs), including Dexamethasone, have been associated with gene regulation for many years. GCs bind a specific receptor (GR), which upon ligation can translocate into the nucleus and bind GC response elements (GREs) to regulate gene expression both positively and negatively. In addition, GR can also regulate gene transcription without binding DNA by directly interacting with other transcription factors and preventing their transcriptional activities (Karin, 1998). GCs have been shown to interfere with AP-1 and inhibit NF-κB (De Bosscher et al., 1997), both of which are important in Th cell cytokine gene expression. VitD3 has also been shown to inhibit the formation of AP-1/NFAT complexes, which results in the repression of *Il2* gene transcription (Alroy et al., 1995), and Dexamethasone signalling results in GR synergising with NFAT to prevent it binding to the *Il4* promoter (Chen et al., 2000b). In combination with APCs, Dexamethasone can induce CD8+ and CD4+ T cells to produce high levels of IL-10 and reduced amounts of IL-4 and IL-5 (Richards et al., 2000). VitD3 has been shown to promote IL-10 production and increase Treg differentiation, while suppressing effector cell proliferation and inflammatory cytokine production (Chambers ES, 2011; Chambers et al., 2014; Jeffery et al., 2009). Together, Dexamethasone and VitD3 can be used to drive a homogenous population of IL-10 producing Th cells that have a regulatory function *in vivo* (Barrat et al., 2002; Xystrakis et al., 2006b).

1.5.2.1.1 T Cell Receptor signalling and regulation of IL-10

Upon TCR ligation a series of signalling cascades are initiated that lead to the activation, translocation or induction of transcription factors. AP-1, NFAT and NF-κB are the main factors that TCR activation induces. AP-1 is downstream of the MAP kinase ERK, which is activated via RAS downstream of the TCR. ERK is thought to be a common factor in different Th subsets that positively regulates IL-10 (Saraiva et al., 2009). AP-1 is a heterodimeric transcription factor composed of dimers of the FOS, JUN, JUN dimerising protein (JDP) and activating transcription factor (ATF) family members (Karin et al., 1997). AP-1, in the form of JUNB and c-JUN, binds at a HSS site within the *Il10* locus in Th2 cells to enhance IL-10 expression. Retroviral expression of this AP-1 complex into naïve CD4+ T cells leads to increased IL-10 expression, and the expression of a dominant negative form of c-JUN decreases IL-10 production (Wang et al., 2005). When Th2 and Th17 cells are stimulated in the presence of IL-21, BATF (another AP-1 family member) can cooperatively bind with IRF4 at CNS-9 in the *Il10* locus (Li et al., 2012a). Furthermore, in the absence of BATF3, BATF can positively regulate IL-10 expression in Th2 cells (Tussiwand et al., 2012). However, BATF3 BATF double knockout Th2 cells also have a defect in IL-4 production, suggesting that BATF3 and BATF may be involved in regulating Th2 differentiation rather that specifically IL-10 expression (Tussiwand et al., 2012). IRF4 has also been shown to positively regulate IL-10 by binding the *Il10* promoter and a CNS within the locus (Ahyi et al., 2009). However, similarly to BATF, IRF4 also regulates IL-4 production and therefore may be involved in Th2 differentiation as a whole and therefore by association IL-10 expression.

TCR activation leads to NFAT1 translocation to the nucleus from the cytoplasm, where it interacts with AP-1 and other partners to promote gene transcription. In Th2 cells NFAT1 has been shown to bind the *Il10* promoter, while in Th1 cells it has been shown to bind in intron 4 of the *Il10* locus (Im et al., 2004). NFAT1 has also been shown to synergise with IRF4 in Th2 cells and bind at CNS-9 to enhance IL-10 expression (Lee et al., 2009a). NFAT blockade reduces IL-10 mRNA in Th1 and Th2 cells, though this also affects the expression of hallmark cytokines, suggesting it may not be an IL-10 specific regulator (Lee et al., 2009a). Though NF-κB is known to be activated downstream of the TCR, and has been shown to be involved in the production of hallmark cytokines from Th1, Th2 and Th17 cells, little is known about its role in IL-10

gene regulation, although it has been reported to bind at the *Il10* gene locus in human T lymphoma cells (Mori and Prager, 1997).

1.5.2.1.2 The effect of cytokines on Th1 cell IL-10 expression

In order to express IL-10 alongside IFNγ, Th1 cells require high levels of antigen stimulation in the presence of IL-12 (Saraiva et al., 2009). IL-12 has also been shown to be important in IL-10 expression in memory Th1 responses, though it has no capacity to induce epigenetic modifications at the *Il10* locus (Chang et al., 2007). Alongside IL-12, much recent evidence has arisen for the role of IL-27 in driving IL-10 expression by Th1 cells. The role of IL-27 in T helper cell differentiation was first noted in 2000, when it was found that IL-27 receptor deficient mice had deficient Th1 IFNγ production when challenged *in vivo* (Chen et al., 2000a), IL-10 production was not measured in this paper. IL-27 augments IL-12 driven IFNγ expression by CD4+ T cells and stimulates the induction of T-bet in a STAT1 dependent manner (Takeda et al., 2003). IL-27 can also signal through STAT3 to drive an IFNγ+ IL-10+ population of Th1 cells (Stumhofer et al., 2007). *In vivo* it has now been shown that IL-27 reduces the severity of EAE by inducing IL-10 expression in CD4+ T-bet+ cells, which in turn downregulates IL-17 expression (Fitzgerald et al., 2007). These IL-27 induced, IL-10 expressing Th1 cells are distinct from Treg cells and do not express FoxP3 (Batten et al., 2008). This year the pathway by which IL-27 drives IL-10 in Th1 cells has been further elucidated. It is now understood that IL-27 triggers two distinct non-redundant pathways in Th1 cells; a STAT1/T-bet pathway and a STAT3/NFIL3 pathway. T-bet and NFIL3 were found to cooperate to drive IL-10 expression alongside Tim-3 expression, a factor associated with T cell exhaustion. These Th1 cells were found to be dysfunctional in the context of gut inflammation and cancer (Zhu et al., 2015). There is some additional evidence that IL-21 can drive IL-10 expression in Th1 cells via a STAT3 dependent manner (Spolski et al., 2009). IL-27 can drive IL-21 expression in Th cells, and therefore it has been suggested that part of the induction of IL-10 by IL-27 may be via IL-21 upregulation.

1.5.2.1.3 The effect of cytokines on Th17 cell IL-10 expression

Th17 cells driven with TGFβ and IL-6, rather than TGFβ, IL-1β, IL-6 and IL-23 have higher levels of IL-10 expression as seen by microarray (Ghoreschi et al., 2010). This is reflected in the phenotypic behaviour of these cells; when driven with TGFβ and IL-6 alone Th17 cells express IL-17 and IL-10 and drive less CNS inflammation. However, IL-10 production by Th17 cells is variable and the cells are heterogeneous. When stimulated with IL-23 the cells express IL-17 and proinflammatory cytokines but not IL-10, leading to a pathogenic function (McGeachy et al., 2007).

Th17 cells cultured with IL-6 and TGFβ *in vitro* can produce large amounts of IL-10, although this is variable. These cultures are heterogeneous and contain three cytokine producing cell types: IL-17+ IL-10-, IL-17- IL-10+ and IL-17+ IL-10+ (Stumhofer et al., 2007). When these cells are cultured with the additional presence of IL-27 the total number of IL-10+ cells is not affected, however the number of IL-17+ cells decreases and the number of IL-10+ IL-17- cells increases (Stumhofer et al., 2007). IL-27 is not reducing IL-17+ cells by initiating IL-10 expression, as even in IL-10 deficient mice IL-27 leads to a decrease in IL-17+ cells. In fact IL-27 and IL-6 can act synergistically with TGFβ to drive IL-10 expression in Th17 cells, and this does not affect FoxP3 expression but does increase IFNγ expression (Stumhofer et al., 2007). IL-27 driven IL-10 depends on STAT1 and STAT3, IL-6 driven IL-10 depends on only STAT3 (Fitzgerald et al., 2007; Stumhofer et al., 2007). It has been suggested that this synergy between TGFβ and IL-27 may initiate the expression of IL-10 via c-Maf, in a STAT1 independent manner (Xu et al., 2009). These findings are reflected *in vivo* where IL-27 deficient mice succumb to excessive inflammation in the autoimmune model of EAE. Furthermore, when adoptively transferring myelin oligodendrocyte glycoprotein (MOG) activated T cells into mice, the transfer of cells cultured in the presence of IL-27 results in reduced disease severity (Fitzgerald et al., 2007). Finally, TGFβ signalling, in the presence of AhR activation has been shown to promote the conversion of IL-17 expressing Th17 cells into IL-10 expressing FoxP3 negative regulatory cells (Gagliani et al., 2015). In summary, the role of IL-27 in the induction of IL-10 by Th cells is complex and remains unclear.

1.5.2.1.4 Cytokine signalling in the regulation of IL-10 in T helper cells

There has been a lot of research into the role of cytokine signalling in IL-10 regulation; with STAT and SMAD proteins downstream of multiple cytokine receptors having been shown to affect IL-10 expression. However, as with TCR signalling, this is closely intertwined with the regulation of Th cell differentiation in general. IL-27 signalling through STAT1 and STAT3 (Batten et al., 2008; Fitzgerald et al., 2007; Stumhofer et al., 2007), IL-21 or IL-6 signalling through STAT3 (Spolski et al., 2009; Stumhofer et al., 2007), IL-12 signalling through STAT4, and IL-4 signalling through STAT6 (Saraiva et al., 2009) have all been implicated in IL-10 regulation in Th cells. STAT4 has been shown to directly bind within the intron 4 of the *Il10* locus in Th1 cells, while STAT6 binds within the promoter region in Th2 cells. STAT4 is thought to promote accessible histone modifications such as H3K4me3 at the *Il10* locus, while STAT6 is thought to antagonise repressive histone modifications such as H3K27me3. Both of these mechanisms increase the accessibility of the locus to transcription factors (Wei et al., 2010). In Th17 cells STAT3 has been shown to bind at intron 4, through its role in IL-10 regulation via chromatin remodelling is yet to be determined (Li et al., 2012a). The role of IL-10 in regulating IL-10 expression, via STAT3, or other mechanisms remains a possibility (Chaudhry et al., 2011). It has been shown that DCs that express high levels of IL-10 can drive Th1 cells to produce IL-10 and take on a regulatory phenotype (Wakkach et al., 2003). Additionally, in the presence of Dexamethasone and VitD3, and in the absence of APCs, T cell secreted IL-10 can act as an autocrine factor to drive further IL-10 expression by these T cells (Barrat et al., 2002; O'Garra and Barrat, 2003).

TGFβ signalling via SMADs can also regulate IL-10 gene expression, though it inhibits Th1 and Th2 differentiation, but not Th17 differentiation (Saraiva et al., 2009). In Th1 cells SMAD4 can bind and transactivate the *Il10* promoter (Kitani et al., 2003). In Th2 cells, GATA3 and SMAD3 can cooperate in response to TGFβ to positively regulate IL-10 expression (Blokzijl et al., 2002). Additionally, as mentioned previously, TGFβ and IL-6 alone can drive Th17 cells to express IL-17 and IL-10 but not proinflammatory cytokines, although this is variable and the cells are heterogeneous (McGeachy et al., 2007).

1.5.2.2 The regulation of IL-10 in T helper cells by Transcription Factors

T helper cell differentiation and IL-10 production are complexly linked; given the important role master transcription factors play in Th cell differentiation, it is possible that these factors may also be involved in regulating IL-10 expression. GATA3 has been shown to bind and remodel the *Il10* gene locus, but alone it cannot transactivate the promoter (Chang et al., 2007; Shoemaker et al., 2006). In differentiated Th2 cells GATA3 is not required for IL-10 production (Zhu et al., 2004), therefore suggesting a role for additional factors in promoting IL-10 expression. The study of STAT deficient Th cells has highlighted a handful of enhancers that are directly regulated by GATA3 or T-bet (Vahedi et al., 2012), or RORγt (Ciofani et al., 2012) in the absence of STATs. Since GATA3 has been shown to directly bind the *Il10* locus, it is possible that T-bet and RORγt may have similar roles in binding and/or altering the accessibility of the *Il10* locus alongside the hallmark cytokine gene loci in Th1 and Th17 cells, respectively. ChIP assays have shown that FoxP3 does not bind directly to the *Il10* locus in Treg cells, but that it does directly target the MAP kinase signalling pathway, which, via AP-1, drives IL-10 expression (Marson et al., 2007; Morikawa and Sakaguchi, 2014).

Data suggests that c-Maf has a role in regulating IL-10 expression in all Th subsets, though the mechanisms by which it does this and the factors that it cooperates with remain elusive and may vary between different cell types. c-Maf is present in all Th cell subsets, albeit at different levels, and its expression has been shown to correlate with IL-10 gene expression. For example, in Th1 cells abrogation of ERK activation leads to a decrease in both c-Maf and IL-10 expression (Saraiva et al., 2009). Furthermore, in Th1 cells that co-secrete IFNγ and IL-10, knockout of c-Maf or AhR correlates with a decrease in IL-10 expression, and both c-Maf and AhR have been shown to bind the *Il10* locus and cooperatively transactivate the promoter (Apetoh et al., 2010). In Th2 cells c-Maf is not thought to be able to transactivate the *Il10* promoter (Kim et al., 1999). However, c-Maf may act in combination with other factors such as AP-1 or NFAT, as it does when driving IL-4 expression (Rengarajan et al., 2002), to regulate IL-10 expression. As mentioned earlier in this chapter both NFAT and AP-1 have been shown to bind the *Il10* gene. In Th17 cells c-Maf, downstream of TGFβ signalling, can induce genes involved in repressing inflammation, such as *Il10* (Ciofani et al., 2012). In fact, in

Th17 cells c-Maf has been shown to bind the *Il10* promoter and transactivate it, to regulate IL-10 expression *in vitro* (Xu et al., 2009).

The E4 promoter-binding protein 4 (E4BP4) is a transcriptional repressor that is encoded by the gene *Nfil3*. E4BP4 has been shown to have a positive regulatory role on IL-10 expression in Th1 and Th2 cells without affecting IFNγ or IL-4 production. The signalling pathway leading to the induction of *Nfil3* in T cells is unknown, however this effect on IL-10 production does not require GATA3, as it is seen in *Gata3* knockout cells (Motomura et al., 2011). In Th2 cells E4BP4 binds the *Il10* gene locus at intron 4 and at the 3' UTR, and may regulate activation state of the *Il10* gene; as in *Nfil3* deficient Th2 cells there is a decrease in histone acetylation of the *Il10* gene locus (Motomura et al., 2011). In Th17 cells, however, overexpression of E4BP4 prevents Th17 differentiation, and therefore there must be different mechanisms regulating IL-10 production in Th17 cells (Ciofani et al., 2012).

TCR signalling can lead to the induction of the *Prdm1* gene, which encodes the PR domain zinc finger protein 1 (known as B lymphocyte induced maturation protein 1; Blimp-1). Blimp-1 is a transcriptional repressor, though it has been shown to positively regulate IL-10 expression in Th cells; conditional deletion of *Prdm1* in T cells leads to pathology and inflammation *in vivo* and decreased IL-10 production by CD4+ T cells (Martins et al., 2006). IL-12 can induce Blimp-1 regulation of IL-10 in Th1 cells *in vitro* and *in* vivo, via STAT4 signalling (Neumann et al., 2014). In this study IL-10 expression in IL-12 driven Th1 cells was dependent on Blimp-1 and additionally enhanced by c-Maf. However, in the presence of TGFβ, IL-10 regulation was shifted to a Blimp-1 independent mechanism that was responsive to IL-27 (Neumann et al., 2014). Nonetheless, IL-27 can also lead to Blimp-1 mediated expression of IL-10 in CD4+ T cells via STAT3 and early growth response 2 (EGR-2). In *Egr-2* deficient cells, IL-27 induction of Blimp-1 and IL-10 expression is impaired alongside dysregulation of IFNγ and IL-17 production (Iwasaki et al., 2013); suggesting EGR-2 may play a role in balancing pro- and anti-inflammatory cytokines. IL-27 has also been seen to drive IL-10 expression via Blimp-1 in CD8+ T cells (Sun et al., 2011). In Treg cells, Blimp-1 synergises with IRF4 to regulate histone acetylation at the *Il10* gene promoter (Cretney et al., 2011). Blimp-1 represses BCL-6, which in turn represses GATA3 (Sawant et al., 2012), so the regulation of IL-10 production by Blimp-1 may be indirect via the

upregulation of GATA3 due to Blimp-1 repressing of BCL-6 (Kusam et al., 2003). However, the processes and mechanisms regulating *Il10* gene regulation remain unclear.

1.5.2.3 Negative regulation of IL-10 in T helper cells

As mentioned above, BCL-6 has been shown to repress GATA3 (Sawant et al., 2012) and therefore may suppress IL-10 via this mechanism. However, BCL-6 has also been shown to suppress IL-10 expression in a GATA3 independent manner; in CD4+ T cells conditionally deficient in BCL-6 there is a dramatic increase in IL-10 expression, but a reduction in Tfh and Th2 cell differentiation, and no effect on Th1 and Th17 differentiation (Hollister et al., 2013). This suppression of IL-10 by BCL-6 is thought to be one mechanism by which it promotes Tfh differentiation. However, as BCL-6 and Blimp-1 reciprocally regulate one another (Johnston et al., 2009), it is possible that BCL-6 suppresses IL-10 expression by suppressing the action of Blimp-1. A final mechanism by which BCL-6 may regulate IL-10 is via the repression of microRNA-21, which has been shown to promote IL-10 expression and is discussed later in this chapter (Sawant et al., 2013). However, the deletion of BCL-6 also leads to increased proinflammatory cytokines, therefore the role of BCL-6 and Blimp-1 in regulating IL-10 and proinflammatory cytokine gene expression in T cells is as yet unclear.

The knockout of the E26 transformation-specific 1 (ETS-1) gene *Ets1* leads to enhanced levels of IL-10 expression in Th cells when cultured in Th1 or Th2 polarising conditions. In Th1 cells, this downregulation of IL-10 by ETS-1 is thought to be via its capacity to maintain the *Il10* locus in a closed confirmation; ETS -1 has been shown to interact with and recruit the histone deacetylase 1 (HDAC1) at the *Il10* gene locus (Lee et al., 2012). However, the role of ETS -1 in Th cell subset differentiation as a whole and its effect on hallmark cytokine expression has not been investigated in detail. It may, however, be essential in Th1 hallmark cytokine expression, as ETS -1 has been shown to synergise with T-bet to positively regulate IFNγ; ETS -1 deletion results in Th1 cells producing less IFNγ and increased amounts of IL-10 (Grenningloh et al., 2005). Another ETS family member, PU.1, has been shown to interfere with GATA3 (Chang et al., 2005) and IRF4 (Ahyi et al., 2009) DNA binding and therefore inhibit production of Th2 hallmark cytokines including IL-10. A direct effect of PU.1 at the *Il10* locus however has not been reported.

1.5.3 The regulation of IL-10 in Macrophages and Dendritic Cells

The combination of several molecular signals determines the production of IL-10 by mDCs and macrophages. Generally stimulation via PRRs is required to express IL-10 (Saraiva and O'Garra, 2010), and co-stimulation of DCs can also enhance their IL-10 production. Additionally, paracrine and autocrine cytokines can drive other signals that modulate IL-10 production by macrophages and mDCs (Gabrysova et al., 2014). The best studied PRRs are the TLRs, of which 2, 4, 5, 7 and 9 have all been shown to induce IL-10 expression in human and mouse macrophages and mDCs (Boonstra et al., 2006; Gabrysova et al., 2014; Kaiser et al., 2009). TLR ligation leads to signalling cascades that are activated through adaptor molecules including myeloid differentiation primary-response protein 88 (MyD88) and Toll/IL-1 receptor (TIR)-domain-containing adaptor protein inducing IFNβ (TRIF). These TLR signalling pathways regulate the expression of TLR-induced cytokines, including IL-10 (Saraiva and O'Garra, 2010).

Signalling through MyD88 activates MAP kinases, such as ERK, and NF-κB; the activation of ERK is thought to be critical to IL-10 production in macrophages and mDCs (Kaiser et al., 2009; McNab et al., 2013). In the absence of ERK, either via chemical inhibitors (Kaiser et al., 2009; Yi et al., 2002) or knockout (Agrawal et al., 2006), TLR activated DCs produce less IL-10. Additionally, the differential levels of ERK activation in innate cells correlates with IL-10 expression; macrophages have the highest levels of ERK activation and have the highest production of IL-10, while pDCs produce no IL-10 and have low levels of ERK activation, mDCs have intermediate levels of ERK activation and produce intermediate amounts of IL-10 (Kaiser et al., 2009). The complexity of IL-10 gene regulation is however paramount; Type I IFN (IFNβ) upregulates IL-10 production in macrophages, whereas TPL2/ERK activation negatively regulated IFNβ whilst upregulating IL-10 (McNab et al., 2015).

When in complex with NF-κB, the tumour progression locus 2 (TPL2) is protected from degradation. Upon TLR activation, TPL2 dissociates from NF-κB and activates ERK. In the absence of NF-κB TPL2 is rapidly degraded and ERK activation is reduced (Beinke and Ley, 2004). TPL2 or NF-κB (Banerjee et al., 2006; Kaiser et al., 2009) deficient macrophages have lower amounts of TLR induced IL-10 production, owing to compromised ERK signalling. However, in NF-κB deficient cells, ERK rescue only

partially restored IL-10 signalling, suggesting ERK-dependent and independent roles for NF-κB in IL-10 regulation (Banerjee et al., 2006). This is supported by evidence that NF-κB can bind directly to the *Il10* gene locus; there is a binding site at a HSS upstream of the TSS where the NF-κB p65 subunit can bind (Saraiva et al., 2005), and NF-κB p50 homodimers have been shown to bind at a site proximal to the TSS (Cao et al., 2006).

Like ERK, the MAP kinase p38 has also been shown to have a role in regulating IL-10 expression in TLR activated macrophages and mDCs. Removal of the dual-specificity protein phosphatase 1 (DUSP1), which negatively regulates p38, leads to prolonged p38 signalling and increased IL-10 production (Hammer et al., 2006). Conversely, chemical inhibition of p38 leads to reduced IL-10 production (Chi et al., 2006). ERK and p38 are also thought to cooperate in their regulation of IL-10, as abrogation of either reduces but does not abolish IL-10 production, whereas abrogation of both completely stops IL-10 expression in TLR stimulated macrophages (Saraiva and O'Garra, 2010). Additionally, the mitogen- and stress-activated protein kinases MSK1 and MSK2, which are activated by a contribution of both ERK and p38, can promote IL-10 expression in TLR stimulated macrophages (Ananieva et al., 2008). The kinase p38 has also been shown to activate mammalian target of rapamycin (mTOR), which is downstream of the PI(3)K-AKT pathway and can promote IL-10 production (Katholnig et al., 2013).

1.5.4 Post-transcriptional regulation of IL-10

There is increasing evidence that in immune cells, IL-10 production is highly controlled at the post-transcriptional level. This regulation is important in ensuring rapid upregulation of cytokine production in response to pathogen invasion, and also in turning off cytokine production to prevent excessive and potentially pathological responses. Many cell types express *Il10* mRNA, however not all of them have detectable amounts of IL-10 protein suggesting that post-transcriptional regulation of IL-10 may be important in many cells (Powell et al., 2000). Most studies looking at *Il10* mRNA stability and post-transcriptional regulation have been done in TLR-stimulated macrophages, therefore other potential mechanisms in different cell types, such as in CD4+ T cells, are still mostly unknown.

The *Il10* gene has a long 3' UTR that contains class II adenosine-uridine-rich elements (ARE) and many potential mRNA destabilising motifs; the half-life of *Il10* mRNA lacking the 3' UTR is significantly longer than that of mRNA with the intact 3' UTR (Powell et al., 2000). These AREs recruit proteins, such as tristetraprolin (TTP), that alter mRNA stability and therefore tend to be a marker of short-lived mRNA (Anderson, 2008). Genome wide analysis has revealed that TTP targets *Il10* mRNA (Stoecklin et al., 2004), and initiates the assembly of RNA decay machinery (Franks and Lykke-Andersen, 2007). Therefore, in TTP deficient macrophages there is a reduced rate of *Il10* mRNA decay and increased IL-10 expression (Gaba et al., 2012; Stoecklin et al., 2004). Furthermore, p38 has been shown to inhibit the action of TTP and therefore stabilise *Il10* mRNA; p38 inhibition enhances TTP activity and its induction of mRNA decay (Tudor et al., 2009). The ARE/poly(U) binding degradation factor 1 (AUF1) binds the 3' UTR of *Il10* mRNA and a reduction in AUF1 levels leads to an increase in *Il10* mRNA half-life (Brewer et al., 2003). In TLR stimulated macrophages AUF1 translocation to the cytosol from the nucleus is thought to be regulated by DUSP1; which explains why in the absence of DUSP1 *Il10* mRNA stability and IL-10 secretion are increased (Yu et al., 2011), although DUSP1 can negatively regulate p38, and therefore may also decrease IL-10 production via this mechanism (Hammer et al., 2006). Alongside being a TTP target, IL-10 can also activate TTP, by binding the TTP promoter and increasing its expression (Gaba et al., 2012), and by reducing p38-mediated inhibition of TTP by acting on p38 to decrease its activity (Schaljo et al., 2009). This suggests there is a feedback mechanism in which IL-10 can limit its own production and therefore maintain an appropriate by balanced immune response.

MicroRNAs (miRNA) have also been implicated in post-transcriptional regulation of cytokines, including IL-10, that are downstream of TLR signalling. The human miRNA-106a has been shown to bind the *Il10* mRNA 3' UTR and induce its degradation (Sharma et al., 2009). In TLR activated macrophages miRNA-466L can upregulate IL-10 production and extend the half-life of *Il10* mRNA by competitively binding at the *Il10* mRNA ARE to prevent TTP binding and therefore preventing TTP-mediated degradation of *Il10* mRNA (Ma et al., 2010). In LPS stimulated macrophages, miRNA-21 has been shown to promote IL-10 production (Sheedy et al., 2010), and recently in Tregs it has been shown that STAT3 upregulates miRNA-21 and in turn

promotes *Il10* expression. However it was suggested that it is possible that this is indirectly via the upregulation of *Gata3* and *Foxp3* (Sawant et al., 2013).

1.6 Using RNA-Sequencing to determine the molecular mechanisms involved in gene expression

The link between the genotype and phenotype of a cell is the result of gene expression. If a molecule of mRNA is present in a cell then the gene for that mRNA must have been transcribed; therefore by looking at what molecules of mRNA are present in a cell one can deduce which genes are being expressed, and depending on the number of mRNA molecules, to what extent. RNA synthesis and maturation are tightly controlled; as it is these RNAs or the proteins encoded by them that drive biological processes. The 'complete complement of mRNA molecules generated by a cell or population of cells' was first termed the transcriptome by Charles Auffary in 1996 (McGettigan, 2013; Pietu et al., 1999). By understanding the transcriptome one can interpret the functional elements of the genome and the molecular constituents of cells and tissues can be revealed.

Transcriptomics is the study of the transcriptome, with the aim of; logging all forms of transcript (including mRNA, non-coding RNA and small RNA), determining the transcriptional structure of genes, and quantifying changes in transcript expression levels in different cell types and under different conditions (Wang et al., 2009). Transcriptome profiling of mammalian cells was first attempted in the early 1990's using automated Sanger sequencing technologies (Adams et al., 1991). Not long after, microarray started being used to deduce the transcriptome (Schena et al., 1995). Microarray is a hybridisation-based technology, in which fluorescent complementary probes of cDNA are used to detect specific mRNA transcripts. This approach is high-throughput and relatively cheap, and dominated the field of transcriptomics for over 10 years. However, as with all technologies, microarray has limitations, including; the reliance on existing knowledge about the genome of interest, high background noise due to cross-hybridisation, and a limited range of detection due to saturation of the probes (Wang et al., 2009). Additionally, to be able to compare across different

experiments the employment of complicated normalisation methods are required. Using sequencing technologies, which directly determine the cDNA sequence, to elucidate the transcriptome were developed at a similar time to microarray (Boguski et al., 1994), but these approaches were low throughput, expensive and not really quantitative. Tagged-based (also known as read-based) sequencing approaches were developed to overcome these limitations as they provide accurate gene expression levels and are high throughput, but this technique was expensive and only a portion of transcripts were analysed (Wang et al., 2009). Now, with the development of new high-throughput DNA sequencing methods, RNA sequencing (RNA-Seq) is supplanting microarray as the choice of technology for gene expression analysis. RNA Sequencing involves the sequencing of complementary DNAs (cDNA) using high throughput DNA sequencing, followed by mapping of these sequence reads to the genome.

1.6.1 RNA Sequencing technology

1.6.1.1 RNA-Seq data generation and analysis

RNA is extracted from a population of cells or tissue, and sheared into smaller molecules that are compatible with sequencing platforms (usually <500 base pairs). These are converted into a library of cDNA fragments, to which DNA adaptors are attached at one or both ends. These adaptors allow the cDNA fragments to be singled out either on beads or on a slide (Marguerat and Bahler, 2010). Each cDNA molecule is then sequenced in parallel in a high-throughput manner, resulting in a series of short sequences that are assimilated into large amounts of data that can be computationally analysed. These resulting short sequence reads, or transcripts, are then aligned to a reference genome or transcripts, or are assembled in a *de novo* manner without a reference genome (Nagalakshmi et al., 2008). For studies of organisms where the reference genome is complete and of high quality, RNA-Seq reads can be directly mapped to the genome, and annotations of that genome can guide analysis and resulting conclusions. Other methods are required when working with organisms that have an absent or incomplete reference genome. From here on I will mainly focus of RNA-Seq data analysis in the context of a model organism, where reads are mapped to a known genome.

Read alignment to the reference genome is the first crucial step in RNA-Seq data analysis, there are many algorithms in circulation that do this (Wang et al., 2009). Prior to alignment, the number of mismatches allowed per transcript and the exclusion of reads that match to multiple regions, must be considered. Reads may not map uniquely for multiple reasons, including; the presence of point mutations, sequencing errors in the transcript, or the read may be from a repetitive sequence (Marioni et al., 2008). Therefore, all of the reads from an RNA-Seq experiment never map uniquely to the reference genome. Once mapped one can computationally generate an expression score for each base that is sequenced, and when comparing that to the reference genome one can establish a high-resolution transcriptome map (Marguerat and Bahler, 2010). From this the overall expression for each gene can be estimated by summing the number of reads that map to exons within that gene. If a change in read count is statistically different between two conditions then the gene can be considered differentially expressed (Chen et al., 2011). Therefore, with RNA-Seq, one can not only quantify gene, and gene isoform, expression, but one can also analyse differentially expressed genes or differential expression between samples. This allows the reconstruction of the whole transcriptome and therefore the transcriptional landscape of a sample at a specific time (Chen et al., 2011). Alongside this, sequencing data can also be used to find novel transcripts and exons within a genome, and it can be used to study alternative splicing (Marioni et al., 2008).

1.6.1.2 The benefits of using RNA-Seq for gene expression analysis studies

RNA-Seq offers advantages and has many benefits over traditional hybridisation based approaches, such as microarray. Firstly, RNA-Seq requires less RNA than microarray, as there are no cloning steps (Wang et al., 2009). RNA-Seq also has lower background noise and higher sensitivity, because sequences of DNA can be mapped to unique genomic locations (Chen et al., 2011). Unlike microarray, RNA-Seq does not saturate, so there is no upper limit for the number of obtainable sequences; it has been estimated for 40 million reads of mouse sequence there is a range of five orders of magnitude over which transcripts can be detected (Marioni et al., 2008). This is close to the estimated range of transcripts found in a cell (Marguerat and Bahler, 2010). Additionally, it has been shown that RNA-Seq produces highly reproducible results with little technical variation (Chen et al., 2011; Marioni et al., 2008; Wang et al., 2009).

RNA-Seq also has a very broad range of applications, with many more on the horizon; it is a technology that is still under development and has not been fully exploited. Unlike microarray, RNA-Seq data can be used to detect novel transcripts and enables the analysis of non-coding regions, as it does not require prior knowledge of the genome. It generates information about what genes are being expressed, and to what level, and about which isoforms of genes are being used by a cell. By knowing the transcripts expressed under certain conditions, studies are aiming to determine the regulatory networks underlying cellular phenotypes. The use of RNA-Seq has already revealed that the complexity of the transcriptome has been hugely underestimated (Marguerat and Bahler, 2010). RNA-Seq is the first technology that enables charting of the entire transcriptome in a quantitative and high-throughput manner. It allows single base resolution and quantification of gene expression on a whole genomic scale, at comparatively low costs (Wang et al., 2010).

1.6.1.3 The limitations and challenges for RNA-Seq

Though RNA-Seq has brought huge benefits to studies and has driven many important discoveries to the field of transcriptomics, it still faces many challenges from both the technological side and the data analysis side. The main technological aspect that limits high quality sequencing data is at the cDNA library construction step. This is a key step as the cDNA library directly mirrors the RNA from the original population, and therefore errors or bias here will be carried through to the rest of the sequencing and analysis process. A robust approach has been developed in which double-stranded cDNA is synthesised from the RNA, to which adaptors are ligated. However, though this approach introduces few errors, it is not ideal for some studies as information about transcriptional direction and transcript orientation are lost (Chen et al., 2011; Marguerat and Bahler, 2010). Methods are now being developed to overcome this drawback, however they are still in initial testing stages and are likely to introduce different biases into the data (Marguerat and Bahler, 2010). Additionally, either the RNA or the cDNA must be fragmented before sequencing can be performed. RNA fragmentation leads to the depletion of fragments ends, while cDNA fragmentation biases for fragments from the 3’ end of a transcript (Wang et al., 2009). Additionally, amplification can lead to polymerase chain reaction (PCR) artefacts, where many copies of certain reads are

found to be present in the library; to overcome this biological replicates are needed to determine if this is an artefact or biologically relevant (Wang et al., 2009). The shotgun process of RNA-Seq, where RNA molecules are randomly fragmented into short sequences, also leads to bias introduction. The number of short reads coming from a transcript is directly related to the length of the RNA molecule; longer molecules break up into more fragments. Therefore the comparison between samples is more efficient for longer transcripts (Oshlack and Wakefield, 2009). Biases in sequences obtained from transcripts have also been found to do with GC content, 3' and 5' end depletion and 3' end bias (Fang and Cui, 2011). Increased samples size or sequence depth is therefore required to overcome these biases, though choosing appropriate protocols and analysis methods can also be very beneficial.

Once samples have been sequenced there are challenges in alignment of the transcripts to the genome. The quality of the output and analysis relies on high quality mapping, and therefore on a high quality reference genome; in non-model organisms this can seriously affect analysis and final conclusions (Chen et al., 2011). Another facet of alignment is that reads can be misaligned if the transcript matches to related genes or other locations within the genome (McGettigan, 2013). Paired end sequencing can help overcome this as it extends the length of the read making it more likely to be specific. Additionally, the introduction of errors means that non-perfect matches may need to be considered when mapping reads. Most algorithms allow for one or two bases mismatching in a read, but deeper sequencing may be needed to resolve larger differences (Wang et al., 2009), particularly if the study is interested in distinguishing SNPs from sequencing errors (Marguerat and Bahler, 2010). Another analysis step that has not been fully eradicated in the transition form microarray to RNA-Seq is normalisation. Though sequencing data needs less normalisation than microarray data, some normalisation and bias correction is still required; at a minimum normalisation for the differences in sequencing depth between each library must be performed (McGettigan, 2013). For differential expression analysis, biases in sequencing depth, distribution of read counts between samples, and the length of genes and transcripts need to be taken into account (Chen et al., 2011).

There are many challenges that still face RNA-Seq at the sample preparation and bioinformatics level. However, this technique has facilitated a huge new area of research and enabled questions to be answered in new and exciting ways. Now lie ahead

not only the refinement of methodologies and analysis, but the application of RNA-Seq to resolve new problems. One exciting feature of RNA-Seq data, that has so far not been applied, is how to use it to identify post-transcriptionally modified or rearranged (and therefore un-mappable) reads (Marguerat and Bahler, 2010). This would greatly increase our understanding of the transcriptome, and therefore the signalling networks and phenotypes, of cells.

1.6.1.4 The application of RNA-Seq to better understand T helper cells

RNA-Seq is a powerful tool to give an overview of the transcriptional profile of different T helper subsets after different treatments or at different stages of differentiation. RNA-Seq can also be used to further refine our understanding of the transcriptional landscape of a cell type; for instance it has been used to discover previously uncharacterised transcripts in mouse naïve and TCR stimulated CD4+ T cells (Hutchins et al., 2012a). However, RNA-Seq can also be used, particularly if in combination with other sequencing techniques, to give a very detailed map of the transcriptional and regulatory networks acting within a cell. Below I discuss three different systems biology approaches that emphasise how RNA-Seq can be and has been used to further our understanding of T helper cell differentiation and transcriptional regulation.

RNA-Seq or microarray provide a temporal snapshot of the transcriptome of a cell. A recent study has used RNA-Seq to look at gene expression dynamics in human Th17 cells over a differentiation time-course (Aijo et al., 2014); with the aim of gaining insight into the differentiation process, and understanding the temporal signatures and dependencies on different factors. They found that *IL17A* expression started to increase above naïve levels after 48hours of culture and continued to increase in a linear manner, while *RORc* took on a more bi-modal expression pattern. Additionally, using this method novel Th17 specific genes were identified. This paper illustrates that RNA-Seq can be used to look at Th cells as they develop and allow one to see how different signalling pathways and regulatory networks are used as differentiation progresses.

However, it is argued that RNA-Seq data alone is not sufficient to fully elucidate the regulatory networks involved in T helper cell differentiation, and that a combination of

techniques are required to establish the transcriptional pathways of these cells. Various studies have tried to combine chromatin immunoprecipitation (ChIP)-Seq with RNA-Seq to give a more comprehensive overview of the regulatory processes within different cell types. Vahedi et al., (Vahedi et al., 2012) used both ChIP-Seq and RNA-Seq of Th cells to link differential patterns of active enhancers, as characterised by the histone modification H3K4me3 and the presence of the acetyltransferase p300, with Th subset specific gene expression. To do this they transcriptionally profiled Th1 and Th2 cells using RNA-Seq, and identified the top 100 differentially expressed genes within each subset. Alongside this they chose to look at 100 housekeeping genes, which were chosen from the literature. Using antibodies to p300 or H3K4me3, they ChIP-Seq'd these cells and compared gene expression to the presence of p300 or H3K4me3 in the gene's promoters. They found very little difference in H3K4me3 enrichment between housekeeping genes and Th subset specific genes. However, p300 was found to be enriched across the whole gene loci of Th subset specific genes compared to housekeeping genes; suggesting that housekeeping genes may be distinctly regulated compared to cell-type-specific genes. Furthermore, they found that expression of genes that were specifically upregulated in one Th subset over the other had significantly higher p300 enrichment. Therefore they concluded that lineage specific p300 binding correlates with cell-type-specific gene expression.

Ciofani et al., published another example of a study comprehensively combining ChIP-Seq and RNA-Seq (Ciofani et al., 2012). In this study they performed ChIP-Seq experiments in Th0 and Th17 cells with antibodies directed against transcription factors known to have a role in Th17 differentiation, to find transcription factor-DNA interactions. They then complemented this data with RNA-Seq on Th17 cells differentiated from naive T cells deficient in the same transcription factors of interest. This resulted in a comprehensive map of where key transcription factors are binding within the genome of Th17 cells and the effects they are having on gene expression. Using an integrated approach they then furthered the study with data from RNA-Seq experiments on 155 T helper cells, and from a public microarray of 167 different immune cell types. The two additional datasets provide regulatory information and further support to the interactions found by the first two datasets. From this they built up a network transcriptional model for mouse Th17 cell differentiation.

1.6.2 RNA-Seq of single cells

RNA-Seq has been a revolutionary step in helping us understand whole transcriptomes and has been used extensively to profile gene expression. However, until the last few years all of these studies have been performed on bulk cell populations or tissues. An average of gene expression is drawn by pooling the RNA from thousands to millions of cells, which can be strongly biased by a few individual cells with very high expression of certain genes; however it may not be reflective of individual cells from within that population (Wu et al., 2014). Even cells with identical genomes have fluctuations in regulatory molecules and gene expression, which can result in significant deviations between cells within a population (Junker and van Oudenaarden, 2014). With recent advances in high-throughput technology, there is now the opportunity to obtain information about the transcriptomes and gene expression profiles of single cells using high resolution RNA-Seq (Tang et al., 2010a; Tang et al., 2010b; Tang et al., 2009).

1.6.2.1 The benefits and limitations of single cell RNA-Seq

Looking at the transcriptome of single-cells is not a new phenomenon. In 1990 a method was developed for the extraction and exponential amplification of cDNA from individual cells (Brady, 1990). Though findings with this technology were very informative, the resolution was limited. Until recently the most viable method for studying single cell gene expression was multiplexed quantitative real-time polymerase chain reaction (qRT-PCR), but the throughput of this technique is limited and biased towards a specific set of genes chosen by the researchers (Saliba et al., 2014). In the 2000s high-density microarray chips paved the way for single cell microarrays, but as mentioned this is hampered by limited dynamic range and sensitivity, and the constraint of only detecting known genes (Tang et al., 2011). Additionally, microarray needs comparatively large amounts of starting RNA, micrograms versus the nanograms needed for RNA-Seq (Saliba et al., 2014). Single cell RNA-Seq overcomes these issues and greatly improves the scope and depth of transcriptome analysis.

Demand for single cell RNA-Seq comes from two viewpoints; firstly for studying populations of rare cells, where the large numbers needed for traditional RNA-Seq are not available; secondly for the analysis of individual cells from heterogeneous

populations. Even in very similar cell types gene expression has been shown to be very heterogeneous and stochastic (Huang, 2009). To evaluate the importance of and to unravel the basis of this heterogeneity, it is necessary to study the transcriptomes of individual cells. Alongside the analysis of differential gene expression, single cell RNA-Seq has highlighted that splicing patterns and differences in allelic expression are highly variable between cells of the same population (Saliba et al., 2014).

However for RNA-Seq of single cells to be successful, various technical and analysis challenges must be overcome. From the technical standpoint; individual cells need to be effectively isolated, minute amounts of cellular RNA need to be converted into cDNA, and this cDNA must be amplified into libraries and sequenced – and all of these techniques need to be successfully combined. Though the amount of starting material required for RNA-Seq is less than that needed for microarray, the material from a single cell still needs to be amplified to create enough for sequencing. This can lead to the accumulation of non-specific by-products and errors during amplification (Kalisky et al., 2011; Tang et al., 2011). From the analysis standpoint; alongside the same challenges faced when analysing all RNA-Seq data there is the additional issue that for statistical significance, many individual cells from a sample must be sequenced (hundreds to thousands) (Saliba et al., 2014). This leads to vast amounts of data, which requires storing, and leads to questions about how to combine the analysis of all these samples efficiently without losing the individual resolution. It is still difficult to distinguish between biological variability and technical noise for low abundance transcripts due to the limited sensitivity of single cell RNA-Seq, which can result in the loss of information about individual transcriptomes (Saliba et al., 2014). Nevertheless this can been overcome (Brennecke et al., 2013) and single cell RNA-Seq on a large number of cells can recapitulate both the bulk transcriptome complexity seen when performing traditional RNA-Seq and the gene expression distributions found in single cells (Wu et al., 2014).

1.6.2.2 Single cell RNA-Seq applications on immune cells

Single cell RNA-Seq can be used to investigate regulatory circuits within cells and explore the heterogeneity in response to stimuli. A recent study from the Regev laboratory (Shalek et al., 2013) investigated the differences between genetically

identical cells at the single cell level by looking at: 1) differences in gene expression between individual cells, 2) differences in expression levels of different transcripts within cells, 3) the splice variants present in the population. They studied *in vitro* generated mouse bone marrow derived DCs (BMDCs) activated with LPS, for two reasons; first they are post-mitotic and therefore lack cell cycle dependent transcriptional differences, and secondly because LPS synchronises cellular responses causing temporal phasing and robust transcriptional responses. The responses of BMDCs to LPS have been studied in detail at the population level and are therefore a good candidate to study at the single cell level. Comparing RNA-Seq data from 18 single BMDCs to data from 3 samples of 10,000 pooled BMDCs, they found that between the 3 pooled samples there was a Pearson's coefficient for gene expression of $R^2 > 0.98$, while for the single cells the average R^2 was 0.48; suggesting extensive cell-to-cell variation. Additionally, splicing patterns were very heterogeneous between the different cells, and generally skewed towards one specific splice variant in each cell. Many of the most highly expressed genes across the single cells showed bimodal expression patterns; being highly expressed in most, but not all, cells. Performing principle component analysis (PCA) on the cells revealed two obvious subpopulations of DC; those with extremely high expression of inflammatory cytokines, and those with weaker expression.

The following year this group published another paper that looked at single cell transcriptomics of 1,700 individual BMDCs (Shalek et al., 2014). Here they found that preventing cell-to-cell communication greatly reduced the heterogeneity of the population, suggesting there is a role for paracrine control in cellular variation. They found that, based on gene expression, a few cells clustered separately from the majority and were in the later stages of maturation. This small subset of cells express a specific set of antiviral genes very rapidly upon stimulation, and produce interferon, which acts in a paracrine manner on the other cells within the population to initiate the expression of the same set of antiviral genes. These studies highlight how looking at covariance between transcripts in individual cells can reveal regulatory circuits that may not be obvious at the population level, and demonstrate the potential role of single cell RNA-Seq in helping to decipher the transcriptome.

A recently study utilised single cell RNA-Seq to elucidate the mechanism of immune suppression by Th2 cells (Mahata et al., 2014). Whole Th1 and Th2 RNA-Seq data

revealed that upon TCR and IL-4 activation, Th2 cells express genes that encode all the factors involved in steroid synthesis. Steroids are known to have immunoregulatory roles (Sakiani et al., 2013), but their de novo production by immune cells has not been investigated. Using single cell RNA-Seq on Th2 cells at different stages of maturation, they found that Cyp11a1, which is the key enzyme in controlling the steroid synthesis pathway, is upregulated as they cells mature. At the single cell level Cyp11a1 expression correlated with many genes involved in Type 2 immune responses, such as *Nfil3, Gata3* and *Il4*, and with numerous factors that have been associated with immunosuppression, including *Il10* and *Tgfb1*. Performing RNA-Seq on the bulk Th2 cell population could not reveal that it is a small subset of the total Th2 cell population that produced these immunosuppressive cytokines along with the steroid pregnenolone. Therefore using single cell RNA-Seq they have demonstrated heterogeneity in the Th2 population, with a previously unidentified subset capable of de novo steroid synthesis and immunosuppression *in vitro* and *in vivo*.

Hematopoietic cells can be used as a model to push the boundaries of single cell RNA-Seq and answer fundamental questions that have so far been restricted due to technical limitations. A key example of this is the massively parallel RNA single cell framework developed in the Amit laboratory (Jaitin et al., 2014). This method has been developed to enable the sampling and transcriptome analysis of thousands of individual *in vivo* cells. As a proof of principle they analysed the *in vivo* transcriptional states of thousands of DCs, a population of cells that is becoming increasingly difficult to characterise into refined cell types based on surface markers. Based on the expression of CD11c they sequenced over 4000 splenic cells, and then classified them firstly on low-depth RNA sampling and secondly on their high resolution transcriptional profiles. A high level of cellular variance was observed, particularly of characteristic cell surface markers, supporting evidence of the high degree of heterogeneity within this population of cells. By pooling the single cell transcriptional profiles they were able to define these cells into splenic subpopulations at a high level of detail, finding significant compatibility with the traditional marker based definitions. Therefore they propose that this method can be used to characterise previously uninvestigated subpopulations of cells within heterogeneous tissues.

1.6.3 The limitations of RNA-Seq of low-quality or low-quantity RNA samples

Single cell RNA-Seq has become more and more viable for analysing the transcriptomes of samples with low RNA quantity, however it is still expensive, and technically and bioinformatically challenging. Therefore several other methods have been devised for overcoming the challenge of low-quantity; two of note are the Ovation RNA-Seq system 'NuGEN' (Head et al., 2011) and the 'SMART' system that switches the 5' end of the RNA template (Islam et al., 2011). Nevertheless, though these have distinct strengths, they also both have weaknesses, and single-cell RNA-Seq is currently overtaking them as a favoured method. Performing high resolution RNA-Seq on low-quality RNA samples, however, is still proving to be a huge challenge. Resolving this issue would be of great importance, particularly for formalin-fixed samples, such as clinical samples that are formalin-fixed paraffin-embedded (FFPE). RNA is a fragile molecule and many biological processes, including ribonuclease digestion, and the fixation of samples can lead to RNA degradation (Opitz et al., 2010), which in turn can prevent efficient ribosomal (r)RNA depletion, reduce library complexity, decrease even transcript coverage of the genome (Adiconis et al., 2013), and lead to biases, such as 3' bias; where transcripts are increasingly degraded from the 5' end (Auer et al., 2003). Loss of 5' ends can inhibit the ability of algorithms to correctly align reads and assemble novel transcripts. Some methods to overcome these issues can alter GC content and transcript length, which in turn create bias, with preferential sequencing of some transcripts over others (Gao et al., 2011; Oshlack and Wakefield, 2009).

Certain assays have been developed to look at partially degraded RNA, such as the Illumina cDNA-mediated Annealing, Selection, extension, and Ligation (DASL) assay (Fan et al., 2004). This is a probe-based assay, where the probes only span 50 nucleotide bases, meaning very short RNA fragments can be assessed. Though this is highly valuable in the clinical setting and can shed light on otherwise un-usable RNA samples (Haller et al., 2006), as with all hybridisation methods it relies on prior knowledge of the genome. Additionally this method doesn't compensate for any bias introduced by the degradation of RNA. Standard microarray has also been used to assess the RNA profiles of FFPE samples, and it has been concluded that the resulting data is comparable to the gene expression profiles seen for identical fresh samples. However, as most microarray probes are designed to ligate the 3' end of a transcript (Opitz et al., 2010), any loss of the 5' end, as expected with 3' bias, will not be seen.

Therefore, though microarray of fresh and FFPE samples may seem equivalent, it is possible information is being lost. In fact it has been shown that when a sample is degraded the likelihood of false positives in differential gene expression is increased (Auer et al., 2003). As RNA-Seq is not probe based, and all transcripts are aligned to the genome, this 3' bias becomes obvious and severely disrupts differential expression analysis (Sigurgeirsson et al., 2014). Therefore RNA-Seq, unlike microarray, can reveal that the RNA of FFPE samples is of low quality. This can be overcome at the analysis stage by computational approaches such as 3' tag counting (Sigurgeirsson et al., 2014), however this only reduces the issue of false positives in differential gene expression. This is still an area that needs much investigation and improvement before RNA-Seq data from low-quality samples can be comparable to results from fresh RNA.

1.7 Project perspective: Using RNA-Seq to elucidate the molecular mechanisms involved in the regulation of *Il10* gene expression

The O'Garra laboratory is interested in understanding the transcriptional networks involved in the regulation of IL-10. Within the laboratory whole populations of multiple T helper cell subsets have been analysed by RNA-Seq and potential factors involved in IL-10 regulation have been discovered, and are now being studied. However, the T helper cell populations are phenotypically heterogeneous, particularly with respect to IL-10 protein production. Usually less than 30% of an *in vitro* cultured T helper cell population will express IL-10, and often less than half will express the hallmark cytokine, and co-expression of IL-10 and the hallmark cytokine is also heterogeneous. Therefore we wanted to devise and implement an innovative new technique that enables RNA-Seq analysis of different cell subpopulations within bulk Th1 / Th2 / Th17 populations based on cytokine protein as seen by intracellular cytokine staining. This technique has never been applied to immune cells and will be greatly beneficial within the field for both *in vitro* and *in vivo* studies. In this thesis I show that it is possible to extract high quality RNA from intracellular cytokine stained subpopulations within Th1 and Th17 bulk populations, even though they are fixed and stained for intracellular cytokines, and that the data from these samples is of high quality and is highly replicable. By separating the subpopulations of different T helper cell subsets according

to their IL-10 expression we have elucidated factors that may be involved in the regulation of IL-10 over those regulating differentiation and hallmark cytokine expression.

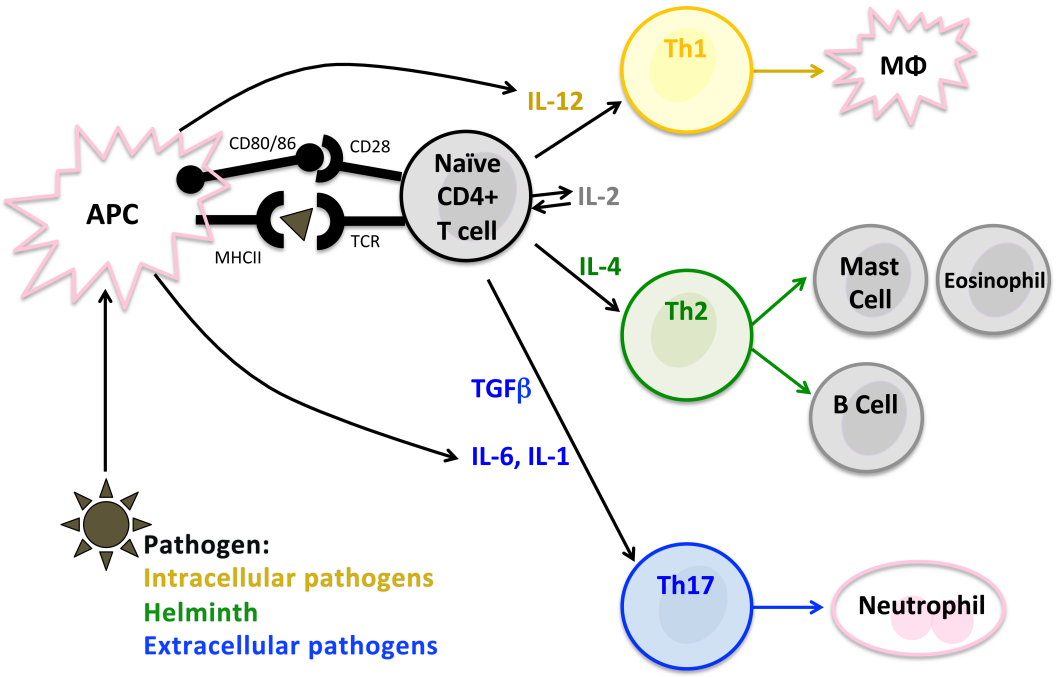


Figure 1.1 Activation and differentiation of T helper cell subsets

Peptides from invading pathogens are presented by APCs to naïve CD4+ T cells in the context of MHC class II. Ligation of the TCR alongside co-stimulatory molecule signaling leads to activation of the naïve CD4+ T cells, resulting in IL-2 production and proliferation. Cytokines in the microenvironment influence the activation of the CD4+ T cells and direct their differentiation into distinct subsets of T helper cell that promote specialised immune responses to eradicate the invading pathogen. MΦ represents macrophages.

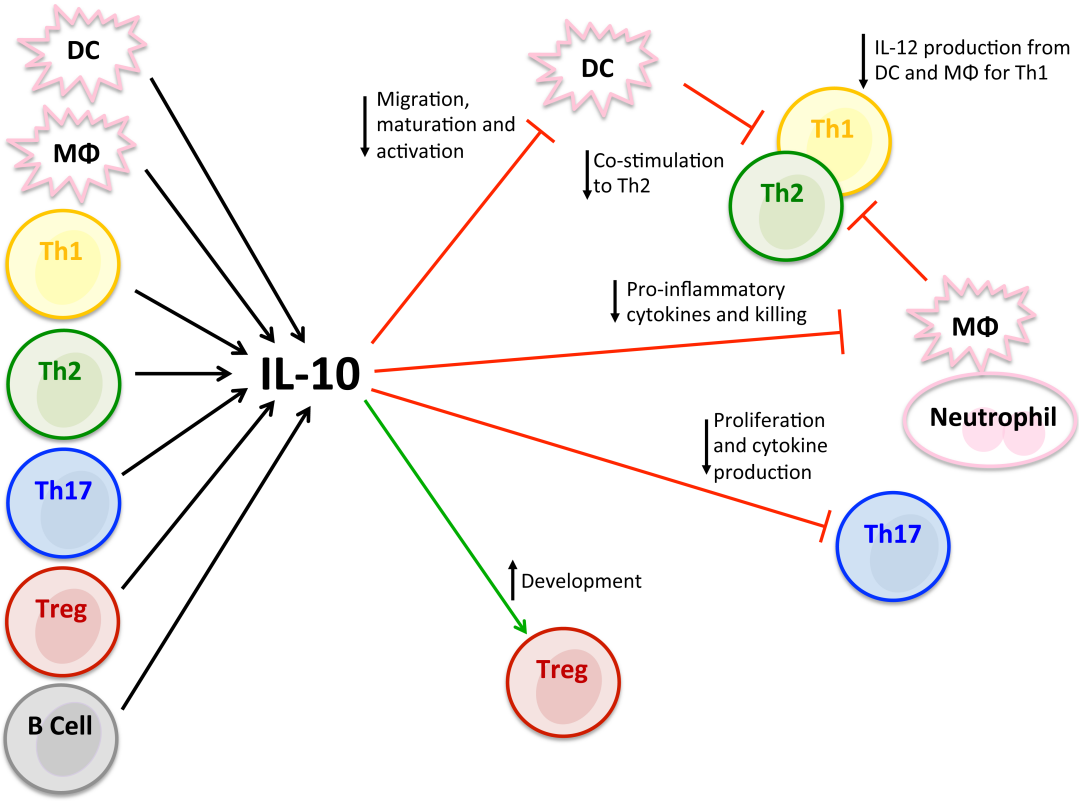


Figure 1.2 The cellular sources and targets of IL-10

IL-10 is produced by many cell of both the innate and adaptive immune systems, and acts predominantly to repress immune responses. IL-10 represses the proliferation and actions of APCs, which in turn indirectly reduces the activation of Th1 and Th2 cells. It also acts directly on Th17 cells to reduce their proliferation and cytokine production. However, IL-10 does also have stimulatory roles; of note it acts directly on Treg cells to promote their development and further IL-10 production. MΦ represents macrophages.

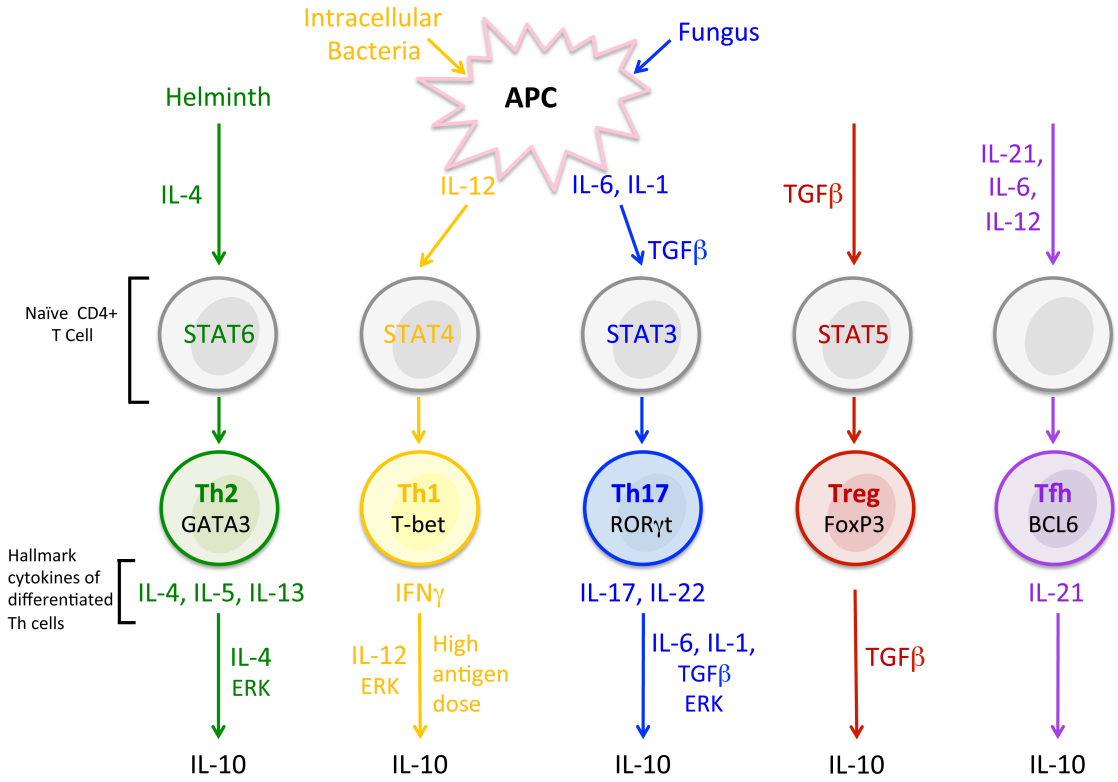


Figure 1.3 Differentiation pathways and IL-10 expression of different Th cell subsets

In accordance with the polarising cytokines, which vary depending on the pathogen invasion, signal transducer and activator of transcription (STATs) and transcription factors are activated in naïve CD4+ T, and act on genes to dictate which cytokines the Th cell produces. IL-10 expression by Th1 cells requires strong antigen dose, conveyed by T cell receptor triggering, and the presence IL-12. Th1, Th2 and Th17 cells require the same factors that are used to polarise them for IL-10 expression. FoxP3^{+} Treg cells require TGFβ *in vivo* to produce IL-10, but other signalling pathways are still unknown. Tfh have been shown to produce IL-10 but the factors needed for this are still poorly understood.

Chapter 2. Materials and Methods

2.1 Mice

C57BL/6 wild type, TCR7(Neighbors et al., 2006), TCR7 Rag KO, FoxP3 KO(Fontenot et al., 2003) TCR7 Rag, GATA3 fl/fl dLck-Cre(Zhang et al., 2005), GATA3 fl/fl(Zhu et al., 2004) mice were bred at the NIMR and maintained under specific pathogen free (SPF) conditions in accordance with the Home Office, UK, Animals (Scientific Procedures) Act 1986.

2.2 *In vitro* differentiation of Th cells

2.2.1 Cell Culture Mediums

RPMI medium: RPMI 1640; 10% heat-inactivated FCS; 0.05mM 2-ME (Sigma-Aldrich); 10mM HEPES buffer; 100U/ml penicillin; 100U/ml streptomycin; 2mM L-glutamine; 1mM sodium pyruvate. Unless stated all reagents purchased from BioWhittaker, Lonza. IMDM medium: Iscove's Modified Dulbecco's Media (Sigma-Aldrich); 5% heat-inactivated FCS; 0.05mM 2-Mercaptoethanol (Sigma-Aldrich); 100U/ml penicillin; 100U/ml streptomycin; 2mM L-glutamine. Unless stated all reagents purchased from BioWhittaker, Lonza. Sort buffer: DPBS with no Ca^{2+} or Mg^{2+} (Gibco); 5% heat-inactivated FCS; 100U/ml penicillin; 100U/ml streptomycin. Unless stated all reagents purchased from BioWhittaker, Lonza.

2.2.2 Isolation of naïve CD4+ T cells

Disaggregated spleens were red blood cell lysed (0.83% ammonium-chloride) and incubated with depletion antibodies against B220 (RA3.6B2 DNAX), MHC class II (M5/114 eBio) and CD8 (C291.2.43 DNAX), T cells were isolated using Goat-anti-rat-IgG heavy chain BioMag beads (Qiagen). Naïve (CD4+CD62L+CD44loCD25-) T cells were isolated by cell sorting with a MoFlo XDP (Beckman Coulter, Inc.) or a FACSAria (BD Biosciences) (**Figure 2.1**).

2.2.3 APC-independent generation of naive activated, Th0, Th1, Th2 cells

Cells were plated at 500,000 cells in 48 well plates and stimulated with plate-bound anti-CD3 (5ug/ml; 2C11 Harlan or 10ug/ml; 2C11 DNAX) and soluble anti-CD28 (2ug/ml; 37.51 Harlan or DNAX). Cells were plated at 500,000 cells per well in 1ml in 48 well plates. Naïve activated cells were stimulated in medium alone. For Th0 induction, cells were stimulated in the presence of anti-IL-4, anti-IL-12, anti-IFNγ, anti-IL-6 and anti-TGFβ (all 10ug/ml). For Th1 induction cells were stimulated in the presence of mouse IL-12 (5ng/ml) and anti-IL-4 (10ug/ml) and in some cases with the addition of IL-27 (2ng/ml). For Th2 induction, cells were stimulated in the presence of IL-4 (10ng/ml) alone or in most cases with the addition of IL-2 (5ng/ml), anti-IL-12 and anti-IFNγ (both 10ug/ml). Cells were cultured at 37^{o}C, 5% CO_2 in RPMI medium. After 3 days of culture cells were removed from the coated wells and split into three. These were then re-plated and given fresh medium and driving cytokines and antibodies as listed above.

2.2.4 APC-independent generation of Th17 cells

For Th17 cell induction, cells were stimulated with plate-bound anti-CD3 (2ug/ml 2C11 Harlan) and plate-bound anti-CD28 (10ug/ml 37.51 Harlan) in the presence of mouse TGFβ (2ng/ml), human IL-6 (50ng/ml) anti-IL-4, anti-IL-12 and anti-IFNγ all at (10ug/ml) and in some cases with the addition of IL-2 (10ng/ml) or anti-IL-2 (10ug/ml). Cells were plated at 500,000 cells per well in 1ml in 48 well plates. Cells were cultured at 37^{o}C, 7% CO_2 in IMDM medium.

2.3 Quantification of cytokine production by ICS of *in vitro* differentiated Th cells

2.3.1 Restimulation of APC-independently generated naive activated, Th0, Th1, Th2 cells

Cells were removed from culture wells, washed with fresh medium and re-plated at 500,000 cells per well in 48 well plates. Cells were restimulated at 37^{o}C for 4h with plate-bound anti-CD3 (2ug/ml) and soluble anti-CD28 (2ug/ml), in the presence of Brefeldin A (10ug/ml Sigma-Aldrich) for the second 2h.

2.3.2 Restimulation of APC-independently generated Th17 cells

Half of the culture medium was removed from the wells, avoiding disturbance of the cells, and an equivalent amount of medium containing PDBu (500ng/ml; Sigma-Aldrich), ionomycin (500ng/ml; Calbiochem) and Brefeldin A (10ug/ml Sigma-Aldrich). Cells were cultured for a further 4h at 37^{o}C.

2.3.3 Preparation of cells for intracellular cytokine stained

After restimulation cells were stained for surface proteins and dead cells using UV LIVE/DEAD fixable dead cell stain (Invitrogen) in PBS with no Ca^{2+} or Mg^{2+} (Gibco); for 30mins at 4^{o}C. Cells were then fixed with 2% formaldehyde (Sigma-Aldrich; diluted from 37-38% in PBS) for cytokine staining for 20mins or with FoxP3 staining buffer set (eBio) for transcription factor staining for 30mins at room temperature. This was followed by permeablisation of cells with permeablisation buffer (eBio) for 30mins at room temperature. Cells were then stained with anti-cytokine or anti-transcription factor antibodies (**Table 2.1**) for 30mins at room temperature in sort buffer. Cells were washed, resuspended in sort buffer and run on the BD LSR II flow cytometer. Data was analysed using FlowJo software (Treestar). Compensation controls were either single colour stained cells from the culture or compensation beads (BD).

Specificity	Function	Cellular Expression	Label	Clone	Supplier	Dilution Factor
IL-2	Proliferation/survival factor	T cells	APC-Cy7	JES6-5H4	BD	500
IL-4	Th2 Cytokine	Th2 cells & B cells	PE	11B11	eBio	200
IL-10	Immunoregulation	T cells, B cells, Macrophages, DCs	APC	JEs5-16E3	eBio	200
IL-17	Th17 Cytokine	Th17 cells	FITC	17B7	eBio	300
IFNγ	Th1 Cytokine	Th1 cells	PE-Cy7	XMG1.2	BD	800
CD4	MHCII co-receptor	Thymocytes, T cells, monocytes, macrophages	E450	RM4-5	eBio	200
			V500	RM4-5	eBio	400
CD8	MHCII co-receptor	Thymocytes, T cells, NK cells, DCs	FITC	53-6.7	eBio	100
CD62L	Adhesion, binds CD34	T & B cells, monocytes, NK cells	PE-Cy7	MEL-14	eBio	400
CD44	Adhesion	T cells, B cells, erythrocytes	PE	IM7	eBio	400
CD25	IL-2Ra chain	Activated T cells and B cells, monocytes	APC	PC61.5	eBio	100

Table 2.1 Antibody details for FACS

2.4 Quantification of cytokine production by ELISA of *in vitro* differentiated cells

2.4.1 Restimulation of APC-independently generated naive activated, Th0, Th1, Th2 and Th17 cells

Naive activated, Th0, Th1 and Th2 cells were removed from culture wells, washed with fresh medium and re-plated at 500,000 cells per well in 48 well plates. Cells were restimulated at 37^{o}C for 48h with plate-bound anti-CD3 (2ug/ml) and soluble anti-CD28 (2ug/ml). The supernatant was then removed from the wells, avoiding disturbance of the pellet, and stored at -80^{o}C for later analysis. For Th17 cells half of

the culture medium was removed from the wells, after differentiation but before restimulation, avoiding disturbance of the cells, and stored at -80^{o}C for later analysis.

2.4.2 Quantification of cytokine production by ELISA

Maxisorp 96-well plates (Nunc, Thermo Scientific) were used for the assay. Commercially available kits were used according to the manufacturer's instructions to quantify the concentration of IL-17 (eBio). Matched-pair sandwich Sandwich ELISAs were used to measure IL-10, IL-4, IFNγ, IL-2 and IL-5 (with antibodies listed in **Table 2.2**). The assay details are summarised in **Table 2.2**. ELISA plates were read on Safire^{2} microplate reader (Tecan). Standard curve calculations and cytokine concentrations were determined using Magellan software (Tecan).

Cytokine	Standard & Starting Concentration	Coating antibody	Detection (biotinylated) antibody	HRP-strepdavidin	Developing substrate
IL-2	Recombinant IL-2 20ng/ml (DNAX)	JES6-IA12 5ug/ml (DNAX)	JES6-5H4 1ug/ml (DNAX)	1ug/ml (Jackson Immunoresearch)	ABTS
IL-4	Recombinant IL-4 20ng/ml (DNAX)	BVD-ID11 4.4ug/ml (DNAX)	BVD6 0.125ug/ml (DNAX)	1ug/ml (Jackson Immunoresearch)	ABTS
IL-10	Recombinant IL-10 (R&D) 10ng/ml	JES5-2A5 5ug/ml (DNAX)	SXC-1 0.25ug/ml (BD)	1ug/ml (Jackson Immunoresearch)	TMB
IFNγ	HDK1 Recombinant IFNγ 50ng/ml (DNAX)	R46A2 2.72ug/ml (DNAX)	AN-18 1ug/ml (DNAX)	1ug/ml (Jackson Immunoresearch)	ABTS
IL-17	10ng/ml	(eBio kit)	(eBio kit)	(eBio kit)	TMB

Table 2.2 Assay details for ELISA

2.5 Quantification of cytokine production by qPCR of *in vitro* differentiated cells

2.5.1 Restimulation of naive activated, Th0, Th1, Th2 cells

Cells were removed from culture wells, washed with fresh medium and re-plated at 500,000 cells per well in 48 well plates. Cells were restimulated at 37^{o}C for the indicated time-points (0, 2, 4, 6, 8, 10, 12 or 24 hours) with plate-bound anti-CD3 (2ug/ml) and soluble anti-CD28 (2ug/ml). Cells were removed from culture wells, washed with PBS and lysed immediately with RLT buffer (Qiagen) containing 1% 2-Mercaptoethanol (Sigma-Aldirch) and lysates were stored at -80^{o}C.

2.5.2 RNA isolation and purification

RNA was isolated according to the manufacturer's instructions using the RNeasy Mini or Micro kit (Qiagen) with an on-column DNase digestion step to remove contaminating DNA (RNase-Free DNase kit, Qiagen). Purified RNA concentration was determined with a Nanodrop spectrophotometer (Nanodrop1000, ThermoScientific).

2.5.3 cDNA preparation and real-time quantitative PCR (qPCR) analysis

cDNA was synthesised from purified RNA using a High Capacity cRNA Reverse Transcription kit (Applied Biosystems). The reaction mixture is summarised in Table 2.3. The following PCR protocol was used to convert RNA to cDNA (Veriti Thermo Cycler, Applied Biosystems): 10mins 25^{o}C, 2h 37^{o}C, 5min 85^{o}C. This was followed by an RNA degradation step in which aDNA was incubated with RNase H (final concentration 0.03U/ul, Invitrogen) at 37^{o}C for 30mins. cDNA was then diluted to 5ng/ul in Nuclease-free H_2O (Preomega). qPCR was conducted using TaqMan Assay system (Applied Biosystems). Reaction mixtures, summarised in **Table 2.4**, were made up in 96-well plates (optical reaction plates, Applied Biosystems), including a no-cDNA template control and a water only control to ensure reagents were not contaminated. The primer-probes used are summarised in **Table 2.5**.

Reagent	Volume	Final Concentration	Source
Primer-Probe	0.5ul	900nM	Applied Biosystems
TaqMan Universal Master Mix	5ul	n/a	Applied Biosystems
cDNA	4.5ul	2.25ng/ul	n/a

Table 2.3 qPCR reaction mixture (per well)

Reagent	**Volume**	**Final Concentration**	**Source**
Cellular RNA	10ul	n/a	n/a
Reverse transcriptase buffer	2ul	n/a	Applied Biosystems
dNTPs	0.8ul	4mM	Applied Biosystems
Ramdom Primers	2ul	n/a	Applied Biosystems
Multiscribe reverse transcriptase	1ul	2.5U/ul	Applied Biosystems
RNasin (Ribonuclease Inhibitor)	0.5ul	1U/ul	Promega
Nuclease-free Water	3.7ul	n/a	Promega

Table 2.4 Reaction mixture for cDNA synthesis

Target Gene	Applied Biosystems code
Il2	Mm00434256_m1
Il4	Mm00445260_m1
Il10	Mm00439616_m1
Ifng	Mm01168134_m1
Il17a	Mm0439619_a1
Hprt	Mm00446968_m1
Gata3	Mm00484683_m1
Tbx21	Mm00450960_m1
Rorc	Mm01261019_g1

Table 2.5 TaqMan primer-probes for aPCR

2.6 Preparation of unfixed samples for RNA-Sequencing

2.6.1 *In vitro* differentiation, restimulation, RNA isolation and purification

Naïve CD4+ T cells were prepared as described in 2.2.2. Th1 and Th2 cells were differentiated as described in 2.2.3 and were harvested on day 7 of culture. Cells were removed from culture wells, washed with fresh medium. 4ml of cells, at ~2.5M cells per ml, were gently layered on top of 3ml of room temperature Ficoll and centrifuged for 45 minutes at 400xg with no braking (Ficoll-Paque Premium 1.084, GE Healthcare). The cell suspension was then taken off the top of the Ficoll and washed with fresh medium. Th1 and Th2 cells were restimulated as described in 2.5.1. RNA was isolated and purified as described in 2.5.2.

2.7 Preparation of cells for Intracellular cytokine staining and Sorting for analysis by RNA-Sequencing

2.7.1 *In vitro* differentiation and restimulation

Naïve CD4+ T cells were prepared as described in 2.2.2. Th1 cells, Th1 + IL-27 and Th2 were differentiated as described in 2.2.3 and were harvested on day 7 of culture. Th17 + IL-2 and Th17 + anti-IL-2 cells were differentiated as described in 2.2.4 and were harvested on day 3 of culture. Th1 cells were restimulated as described in 2.3.1. Th17 were restimulated as described in 2.3.2.

2.7.2 Fixation, Intracellular cytokine staining and Sorting

After incubation cells were stained for the surface protein CD4 and dead cells using e450 LIVE/DEAD fixable dead cell stain (Invitrogen) in PBS with no Ca^{2+} or Mg^{2+} (Gibco); for 30mins at 4^{o}C. Cells were then fixed with 4% formaldehyde (Sigma-Aldrich; diluted from 37-38% in PBS) containing 0.4U/ul RNasin Plus (Promega) for 30mins at 4^{o}C. Cells were then washed with PBS containing 20% sort buffer and 0.4U/ul RNasin Plus. This was followed by permeablisation of cells with permeablisation buffer (eBio) containing 1.6U/ul for 15mins at 4^{o}C. Cells were then stained with anti-cytokine antibodies (**Table 2.1**) in permeablisation buffer (eBio) containing 1.6U/ul for 30mins at 4^{o}C. Cells were then washed with PBS containing 20% sort buffer and 0.4U/ul RNasin Plus. Cells were then resuspended in PBS containing 10% sort buffer and 1.6U/ul RNasin Plus. Samples were then run through the FACSAria cell sorter (BD Biosecinces) using FACSDiva software, and collected into FACS tubes with 500ul of PBS containing 10% sort buffer and enough RNasin Plus to result in a concentration of 1U/ul when the tube was filled to 5ml. Compensation controls were single colour stained cells.

Four subsets of Th cells were sorted, each with 3 biological repeat experiments; Th1, Th1 + IL-27, Th17 + IL-2 and Th17 + anti-IL-2. These subsets were sorted as live CD4+, to collect the 'bulk' population of cells, and into their different intracellular cytokine producing subpopulations (**Figure 2.1)**. The 3 biological repeat experiments

had similar percentages of live CD4+ cells in each intracellular cytokine producing subpopulation (See Chapter 5 & 6).

2.7.3 RNA isolation and purification

After sorting, cells were pelleted by centrifugation at 3000 g for 5mins at 4^{o}C. The supernatant was discarded. Total RNA was isolated from the pellet using the FFPE RNeasy Kit (Qiagen), starting at the protease digestion stage of manufacturer-recommended protocol. The following modification to the isolation procedure was made: instead of incubating cells in digestion buffer for 15 minutes at 50^{o}C and 15 minutes at 80^{o}C, we carried out the incubation for 3 hours at 50^{o}C. Cell lysates were frozen at -80^{o}C overnight before continuing the RNA isolation by the manufacturer's instructions. Purified RNA concentration was determined with a Nanodrop spectrophotometer (Nanodrop1000, ThermoScientific).

2.8 RNA-Sequencing

2.8.1 Verification of RNA Quality

The quality of RNA was determined using an Agilent 2100 Bioanalyser (Agilent Technologies). All RNA samples that were not part of the optimisation steps had a RNA integrity number (RIN) over 7, meaning RNA was not degraded and of high quality (Schroeder et al., 2006).

2.8.2 Preparation of samples for RNA-Sequencing

RNA was obtained from 3 biological replicates of differentiated T helper cells. The samples were processed for RNA-Seq by the Francis Crick Institute Mill Hill laboratory's HTS facility. Samples were poly-A purified and converted to cDNA libraries using the Illumina TruSeq Library preparation kit v2. 10-12pM of samples were multiplexed 6 to 7-per-lane for sequencing using the Illumina HiSeq 2500 platform with single-end read lengths of 50bp, resulting in 27M to 65M reads per sample, and an average biological fragment length of 301-328 nt.

2.8.3 Analysis of RNA-Sequencing data

2.8.3.1 Pre-analysis processing and normalisation

Reads were aligned to the mouse transcriptome and genome (GRCm38 / mm10, 2014.10.08) using Strand NGS (Version 2.0) guided by RefSeq annotations (2013.04.01) (Pruitt et al., 2014; Tatusova et al., 2014), with 95% identity, max 5% gaps, 1 read only if duplicated. Samples were normalised using the count based technique DeSeq (Anders and Huber, 2010); with no normalisation to the baseline in Chapter 3 and initial quality controls in Chapter 5 & 6; with normalisation to the baseline median of the samples, for further analysis in Chapter 5 & 6. Results are outputted as a Log2 value.

2.8.3.2 Relative gene coverage

Aligned BAM files were exported from Strand NGS, in bash these were sorted and indexed using SAMtools (Li et al., 2009). The output was analysed for relative gene coverage over the mouse transcriptome (RefSeq, mm10) using the python module geneBody_coverage.py, which was imported from the RSeQC package (Wang et al., 2012). This module is used to check if reads coverage was uniform and if there was any 5' or 3' bias.

2.8.3.3 Hierarchical clustering

All hierarchically clustered according to conditions or conditions and entities used Pearson's centred distance metric with Average linkage rule, in Strand NGS software. Hierarchical clustering constructs a dendrogram in which entities are represented in a relationship tree that allows the visualisation of the data within one heat map. This groups samples based on their similarity, and therefore similar samples should cluster together. Gene expression was shown as a red-blue heat map, with red indicating upregulation, blue indicating downregulation and yellow no change. The Pearson centred distance metric was used as this clusters genes based in the expression profile of

the entities over the different samples rather than by the magnitude of gene expression which is used by Euclidean-based distance metric. We were more interested in genes that had similar expression patterns as they are more likely to be co-regulated, regardless of the magnitude of induction. Groups of genes were manually curated based on dendrogram and experimental hypotheses.

2.8.3.4 Fold change

For all fold change the data from the three repeats of each sample was pooled. Differentially regulated genes were obtained by taking those that were at least 2 or 3-fold up- or downregulated in at least 1 of the samples vs. the baseline (median of all the samples). Log fold change is the difference between two Log values; which is equal to the fold change difference between two raw read values.

2.8.3.5 Pathway and network analysis with IPA

Pathway and network analyses were conducted using Ingenuity Pathway analysis (IPA) (Ingenuity® Systems, www.ingenuity.com). Ingenuity relies on information from the manually curated database Ingenuity Knowledge Base (Calvano et al., 2005). Significant association to a gene list was determined by Fisher's Exact Test. IPA was used to determine signalling pathways and gene netwroks associated with clusters of differentially expressed genes. Interactions were considered significant if $p<0.01$.

2.8.3.6 Functional analysis with GO

Functional analysis was carried out using gene ontology (GO) analysis in Strand NGS. In the GO database genes are assigned ontologies relating to their molecular functions, associated biological processes and cellular locations (Harris et al., 2004). Lists of genes can be analysed regarding their associated GO ontologies and significant associations with particular terms can be calculated.

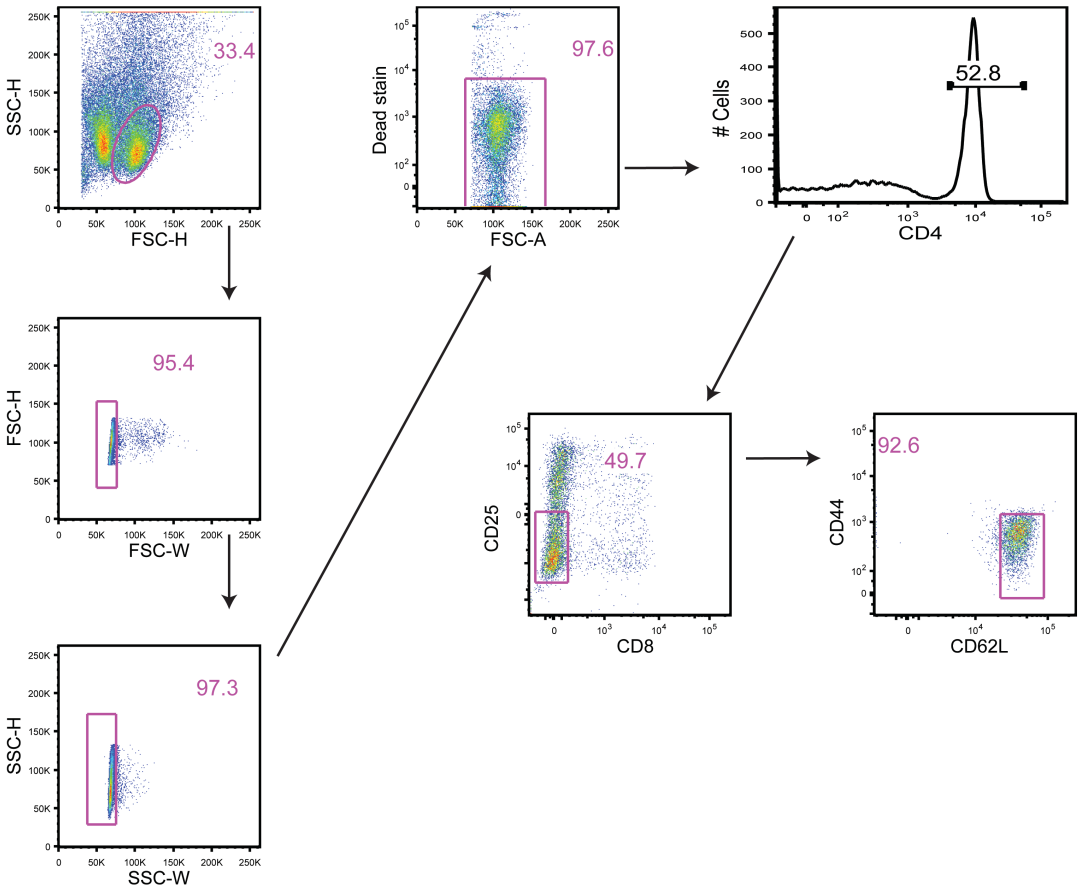


Figure 2.1 Gating strategy for separation of naive CD4+ CD62L + CD44 Low CD25- cells

Single cell suspensions from mouse spleens were generated as described in section 2.2.2. Naïve (CD4+CD62L+CD44loCD25-) T cells were isolated by cell sorting with a MoFlo XDP or a FACSAria. Gates indicate the percentages of parent. Data shown are from one experiment. FSC = forward scatter; SSC = side scatter. Other information relating to fluorochrome-conjugated antibodies may be found in Table 2.1.

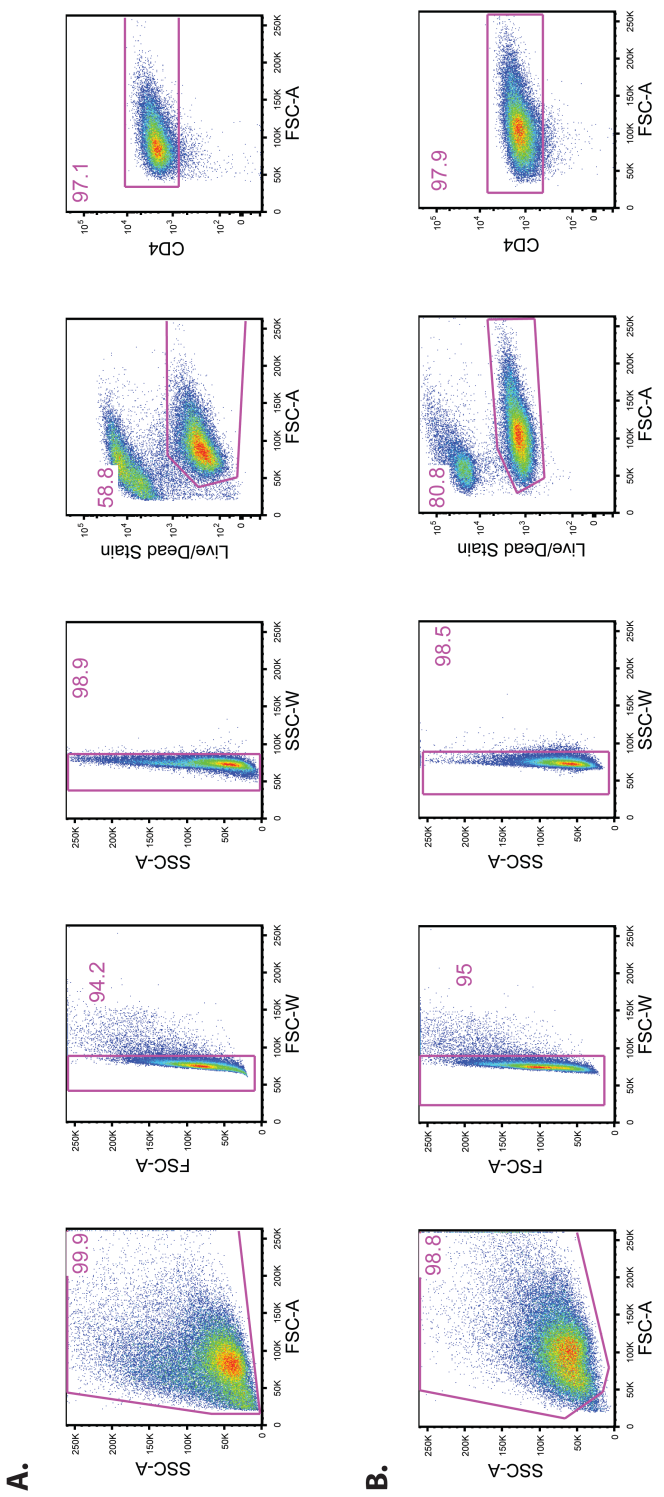


Figure 2.2 Gating strategies for separation of live CD4+ Th1 and Th17 cells for RNA-Seq analysis

A. Th1 cells and Th1 + IL-27 cells and **B.** Th17 + IL-2 cells and Th17 + anti-IL-2 cells were prepared for fixation, intracellular cytokine staining and sorting as describes in section 2.7.2. Gates indicate the percentages of parent. Data shown are from one experiment. FSC = forward scatter; SSC = side scatter. Other information relating to fluorochrome-conjugated antibodies may be found in Table 2.1.

Chapter 3. Optimisation of the *in vitro* differentiation of IL-10 secreting T helper cells

3.1 Background

IL-10 is an immunoregulatory cytokine that has a vital role in maintaining a balanced and appropriate immune response. It is fundamental that this balance between inflammatory responses required to eradicate a pathogen, and excessive inflammation which would harm the host, is maintained. CD4+ T helper cells are important in regulating an effective immune response, and are a dominant source of IL-10 that is crucial in regulating T cell responses (Roers et al., 2004). Despite the different signalling pathways that result in the polarisation of each Th subset and lead to the expression of their hallmark cytokines, IL-10 is expressed by all of the different Th subsets (Saraiva and O'Garra, 2010). Current studies suggest that the differentiation pathway of each T helper subset, governed by a set of distinct master transcription factors, is tightly linked to IL-10 expression (Gabrysova et al., 2014). It is therefore likely that there are disparate, but potentially overlapping, mechanisms by which IL-10 expression is regulated in each Th subset.

CD4+ T cells require TCR stimulation and signalling from cytokine receptors to fully differentiate and they will only express cytokines, other than IL-2, once they have differentiated (Yamane and Paul, 2013). In the absence of cytokine signalling CD4+ T cells will become activated and express IL-2, which acts in an autocrine manner to promote proliferation, but will not differentiate into a Th cell subset (Murphy et al., 1996; Shoemaker et al., 2006). Once Th cells have differentiated they express their hallmark cytokines, in addition to IL-10. Each Th subset requires different types of TCR and cytokine signalling to drive differentiation and IL-10 expression. Th1 cell differentiation is driven by IL-12. However, it has been shown that strong TCR ligation is required to initiate IL-10 expression in this cell type (Saraiva et al., 2009). Additionally, IL-27 has been shown to boost IL-10 expression in Th1 cells (Stumhofer and Hunter, 2008). Th2 cells require IL-4 to drive the production of their hallmark cytokines and IL-10 (Fiorentino et al., 1989). Th17 cells, primarily driven by TGFβ and IL-6, express IL-10 transiently and variably, via mechanisms that are still unclear. Though IL-23 has been shown to suppress IL-10 expression (Ghoreschi et al., 2010; McGeachy et al., 2007). Therefore, for optimal IL-10 expression alongside hallmark cytokine expression, different Th subsets appear to require different specific signals and different times of stimulation. This is particularly difficult to mimic *in vitro* as slight

alterations in dose of TCR ligation or cytokine signalling can have profound effects on T cell differentiation and cytokine expression (Constant et al., 1995; Gabrysova et al., 2011; Gabrysova and Wraith, 2010; Hosken et al., 1995; O'Garra et al., 2011). Therefore, we optimised our *in vitro* systems to study hallmark cytokine and IL-10 co-expression in Th1, Th2 and Th17 cells subsets.

Within the O'Garra laboratory a protocol for differentiating Th cells *in vitro* in an APC independent manner has been optimised. This involves the antibody anti-CD3, which stimulates the TCR by binding the protein CD3, and anti-CD28, which binds to CD28 and costimulates the T cell resulting in additional signals for T cell activation. This method of T cell stimulation benefits from being able to stimulate TCRs of any specificity, and therefore transcription factor knockout mice can be used without the need to be crossed with a TCR transgenic line. Furthermore it is the only form of stimulation that can be used on human T cells. Using this system one can determine direct effects on T cells without the involvement of additional factors from APCs.

The duration of CD4+ T cell culture *in vitro* and the number of times a cell is stimulated via the TCR and cytokine receptors can profoundly affect the concentration of cytokines produced (Aijo et al., 2014; O'Garra et al., 2011). Furthermore, it has been highlighted that once stimulated *in vitro*, Th cells only produce small amounts of their hallmark cytokines unless they are restimulated (Murphy et al., 1996). This restimulation can be induced with either TCR activation, such as with anti-CD3 and anti-CD28, or with a mix of a protein kinase C (PKC) activator, such as phorbol 12,13-dibutyrate (PDBu) or phorbol 12-myristate 13-acetate (PMA), and a calcium mobiliser, such as Ionomycin (Iono). Therefore to study cytokine production from Th cells, one must restimulate the cells after culture; no doubt the kinetics of this restimulation are also important in capturing optimal cytokine expression.

To study optimal cytokine production from Th cells they must be differentiated and restimulated after culture. The kinetics of both differentiation and restimulation are important in capturing maximal cytokine expression. Thus, the conditions under which naïve CD4+ T cells are polarised into different Th subsets have a fundamental impact on cytokine expression. The type of TCR stimulation, the cocktail and dose of polarising cytokines, the length of culture, and the duration of restimulation all play an

important role in determining IL-10 and hallmark cytokine production, and were optimised for our study.

3.2 The optimisation of *in vitro* differentiation of IL-10 secreting T helper cells: Study Aims

To better characterise the regulation of IL-10 gene expression in Th cells, we needed to be able to differentiate Th cells that express their hallmark cytokines and IL-10. Therefore it was fundamental for future experiments that a system for differentiating IL-10 producing Th cell subsets *in vitro* was optimised. With this intention, we set out to answer the following questions:

1. How long do Th cells need to be cultured *in vitro* for optimal IL-10 expression alongside expression of hallmark cytokines?
2. What is the optimal restimulation duration for analysis of IL-10 mRNA from differentiated Th cells?

3.3 Results

3.3.1 Determining the optimal duration of *in vitro* culture of Th cells for hallmark cytokine and IL-10 production

We wanted to assess the kinetics of hallmark cytokine and IL-10 production from Th1, Th2 and Th17 cells stimulated *in vitro* with anti-CD3 and anti-CD28, to evaluate the best duration of culture for each Th subset.

3.3.1.1 Different Th cell subsets produce optimal amounts of hallmark cytokines and IL-10 after different durations of differentiation

Naïve CD4+ T cells were stimulated with anti-CD3 and anti-CD28 in the presence of Th1, Th2 or Th17 driving cytokines and blocking antibodies as described in the Materials and Methods. Th1 and Th2 cells were cultured for 1, 2, 3, 5 or 7 days. Th17 cells were cultured for 1, 2, 3, or 5 days, as Th17 cells were not split during culture, they do not survive beyond 5 days. Samples were stained for intracellular IL-2, IL-10 and their associated hallmark cytokine (**Figure 3.1**). During the 7-day time course of Th1 cell differentiation IL-2, IL-10 and IFNγ production increased. Between day 3 and day 5 there was a dramatic increase in production of all three cytokines, and between day 5 and day 7 the amount of each cytokine produced almost doubled; with maximal amounts of IL-10 and IFNγ being produced at day 7 (**Figure 3.1**). A similar kinetic of IL-2, IL-10 and IL-4 cytokine production was seen for Th2 cells; with maximal amounts of IL-10 and IL-4 being produced at day 7 (**Figure 3.1**). In contrast Th17 cells showed a very different cytokine production profile. Th17 cells produced large amounts of IL-2 at day 1, which dramatically decreased by day 2, and then gradually increased again until day 5; this profile was mirrored in levels of IL-2 mRNA (data not shown). IL-17 production gradually increased over the time-course, while IL-10 production peaked at day 3 and then decreased again by day 5 (**Figure 3.1**). Therefore, unlike in Th1 and Th2 cells, IL-10 production by Th17 cells does not increase in parallel with hallmark cytokine production.

Alongside intracellular cytokine staining, IL-10 and hallmark cytokine mRNA expression was assessed (**Figure 3.2**). In Th1 and Th2 cells *Il10* mRNA expression steadily increased over the 7-day period. In contrast Th17 *Il10* mRNA expression peaked at day 2 and then decreased again by day 5. As IL-10 protein is translated from mRNA, it is not surprising that in Th17 cells *Il10* mRNA peaked the day before the protein levels peaked (**Figure 3.1**). Th2 cells had the highest levels of *Il10* mRNA at day 7, while the day 2 levels of *Il10* mRNA expressed by Th17 cells were greater than the maximum *Il10* mRNA levels seen for Th1 cells at day 7 (**Figure 3.2**). *Ifng* expression in Th1 cells peaked at day 3; this does not mirror the increasing IFNγ protein levels seen over the 7 days (**Figure 3.1 & 3.2**). This may explained by the fact that when analysing intracellular cytokines the cells are restimulated, and *Ifng* mRNA in Th1 cells may not be constitutively expressed, but upon TCR signalling it may be dramatically upregulated. In Th2 cells *Il4* mRNA expression peaked at day 5 and stayed high at day 7 (**Figure 3.3**). In Th17 cells *Il17* mRNA reflected the protein by gradually increasing to a peak at day 5 (**Figure 3.1 & 3.2**).

To further analyse the kinetics of the Th cell subset differentiation, the kinetics of master transcription factor mRNA expression were assessed (**Figure 3.3**). In all three Th cell subsets the levels of master transcription factor expression increased over the culture period. In Th1 cells T-bet mRNA (*Tbx21*) expression was very low at day 0 in naïve cells, with a gradual increase to day 7 (**Figure 3.3**). *Gata3* expression in day 0 naïve T cells was relatively high, which is not surprising as GATA3 is expressed throughout T cell development (Wei et al., 2011). In Th2 polarising conditions *Gata3* mRNA expression gradually increased to day 5 (**Figure 3.3**). In naïve T cells *Rorc* mRNA is almost undetectable, however upon stimulation in Th17 driving conditions *Rorc* levels were dramatically increased and peaked at day 3 where levels plateau (**Figure 3.3**).

Therefore the day at which optimal concentrations of IL-10 and hallmark cytokines are produced differs between the different Th cell subsets. Additionally the mRNA profiles and protein profiles do not always follow the same trend, often, as expected, with mRNA peaking before protein. For future experiments we were interested in looking at both mRNA and protein and therefore a balanced time-point was chosen for the optimal duration for culture and when the highest levels of IL-10 were observed.

Il10 mRNA and intracellular protein production by Th1 and Th2 cells was optimal at day 7 (**Figure 3.1 & 3.2**). *Il4* and *Gata3* mRNA in Th2 cells peaked earlier than intracellular IL-4 protein, but mRNA levels remained high at day 7 (**Figure 3.1, 3.2 & 3.3**). Though *Ifng* mRNA expression decreased by half after day 3 of Th1 culture, *Tbx21* mRNA expression and IFNγ protein production peaked at day 7 (**Figure 3.1, 3.2 & 3.3**). *Il10* mRNA and intracellular protein in Th17 cells peaked at day 2 during the cell's differentiation, while *Il17* mRNA and intracellular protein and *Rorc* mRNA production were optimal at day 5 (**Figure 3.1, 3.2 & 3.3**). Taking all this information into account, and with importance placed on the further study of IL-10 expression alongside the expression of hallmark cytokines, the optimal duration for Th1 and Th2 cell culture was chosen as 7 days, while for Th17 cells as 3 days.

3.3.1.2 Differentiated Th1, Th2 and Th17 cells are distinctive from naïve activated and Th0 cells and do not produce reciprocal hallmark cytokines

To further define Th1 and Th2 cells differentiated for 7 days, and establish their cytokine expression profiles, we compared them to *in vitro* cultured 'naïve' cells. As previously described, in the absence of cytokine signalling CD4+ T cells become activated and express IL-2, but do not differentiate into a Th cell subset (Murphy et al., 1996; Shoemaker et al., 2006). Our *in vitro* cultured 'naïve' cells mimic this phenotype and were differentiated in culture via two methods; naïve activated cells represent naive CD4+ T cells that are stimulated with anti-CD3 and anti-CD28 but are not driven with any polarising cytokines or blocking antibodies; Th0 cells represent naïve CD4+ T cells that are stimulated with anti-CD3 and anti-CD28 and cultured in the presence of blocking antibodies for IL-4, IL-6, IL-12, IFNγ and TGFβ.

Naive activated cells, as expected, only produced IL-2 intracellular protein (**Figure 3.4**) and mRNA (**Figure 3.5**). Interestingly, naive activated cells, which are not repressed by any blocking antibodies, also made minimal amounts of IFNγ protein alongside IL-2 (**Figure 3.4 & 3.5**) suggesting the population may have contained some Th1 cells under these conditions, though the amounts were minimal compared to those seen from Th1 cells. Th0 cells also produced very large amounts of IL-2 and no IL-10, IL-17 or IFNγ (**Figure 3.5**). However, they made small amounts of IL-4 at the protein level (**Figure 3.4 & 3.5**), suggesting they may have contained some Th2 cells under these

conditions, though the amounts were again insignificant compared to those seen from Th2 cells.

Th1 cells produced large amounts of their hallmark cytokine IFNγ and there was a robust Th1 population producing IL-10 (**Figure 3.4 & 3.5**). Of note, IL-10 production was only seen in the IFNγ positive population of cells (**Figure 3.4**). This is supported by the literature that suggests Th1 cells make IL-10 to feedback and inhibit production of IL-12 by APCs to control themselves and promote anergy (O'Garra and Vieira, 2007; Saraiva et al., 2009). Therefore, in this APC free culture system this IL-10 will not be having an effect on the Th1 cells. Th1 cells did not produce IL-4 or IL-17 protein (**Figure 3.4 & 3.5**). This highlights that they are distinctive from the Th2 and Th17 cells and behave as characteristic *in vitro* cultured Th1 cells. Th2 cells made large amounts of the hallmark cytokine IL-4 and substantial amounts of IL-10 (**Figure 3.4 & 3.5**). Unlike the Th1 cells, Th2 cells produced IL-10 alongside IL-4 and there was a distinct population of IL-10+ IL-4- cells within the Th2 subset (**Figure 3.4**). Th2 cells did not produce IFNγ or IL-17 protein (**Figure 3.4 & 3.5**). Th17 cells produced large amount of the hallmark cytokine IL-17, as seen by ICS (**Figure 3.4**), however the amount of IL-17 as seen by ELISA (**Figure 3.5**) was much less than the amount of IFNγ produced by Th1s or IL-4 produced by Th2s; possibly because Th17 supernatants were taken prior to restimulation. Like Th2 cells, Th17 cells produced IL-10 alongside IL-17 and there was a distinct population of IL-10+ IL-17- cells within the Th17 subset (**Figure 3.4**). This may be due to the fact that, unlike Th1 cells, Th2 and Th17 cells can respond to extracellular IL-10 (Coomes, In Press. ; Huber et al., 2011). Th17 cells did not produce IFNγ or IL-4 protein (**Figure 3.4 & 3.5**). Furthermore, none of the naive activated, Th0, Th1, Th2 or Th17 cell populations expressed the Treg master transcription factor FoxP3 (data not shown). Therefore, after 7 days of *in vitro* culture of CD4+ T cells in Th1 or Th2 driving conditions, or after 3 days in Th17 driving conditions, we are confident that these cells have differentiated from naïve cells. They have robust hallmark cytokine expression phenotypes that are distinctive from each other, and yet the Th1, Th2 and Th17 populations all produce IL-10.

3.3.2 Determining the optimal restimulation duration of differentiated Th cells for hallmark cytokine and IL-10 production

We have established that stimulating naïve CD4+ T cells with anti-CD3 and anti-CD28, and differentiating them for 3 days for Th17 cells and 7 days for Th1 and Th2 cells, is the best method for optimal hallmark cytokine and IL-10 production. Now we sought to assess the kinetics of hallmark cytokine and *Il10* mRNA expression after restimulation of differentiated Th1 and Th2 cells to evaluate the best restimulation time for analysis. This analysis was not performed on Th17 cells as the different differentiation timings meant the analysis could not be run in parallel. However, a microarray analysis run in the O'Garra lab of mRNA kinetics from Th1, Th2 and Th17 cells (Gabrysova, Unpublished) supported the data seen here and found similar Th17 hallmark cytokine and *Il10* kinetics as those seen for Th1 and Th2 cells.

3.3.2.1 Th1 and Th2 cells express optimal amounts of mRNA of IL-10 and hallmark cytokines after 4 to 6 hours of restimulation

As we were fundamentally interested in gene expression, not only did we need to choose an optimal time for analysing Th cells based on intracellular protein production, but also on mRNA expression, as this is the best representation we have for gene expression. To determine the time points at which *Il10* and hallmark cytokine mRNA expression peaked, we performed a kinetic of restimulation of differentiated Th1 and Th2 cells over a 24 hour period (**Figure 3.6**). Th1 cells expressed *Ifng*, *Il10* and *IL2* mRNA and Th2 cells expressed *Il4*, *Il10* and *Il2* mRNA, while naive activated and Th0 cells only expressed *Il2* mRNA. In all the cell types *Il2* dramatically increased to a peak at 4 hours, and then sharply decreased again to minimal amounts by 8 hours (**Figure 3.6**).

In Th1 cells, *Il10* peaked at 4 hours and is maintained until 6 hours of restimulation, from which point it gradually decreased. *Ifng* was not expressed at substantial levels until 2 hours post restimulation, at which point it was rapidly expressed, peaking at 6 hours, from which point expression considerably decreased again (**Figure 3.6**). Of note, the starting amounts of *Ifng* mRNA at 0 hours of restimulation were comparable to the

levels seen before when Th1 cells were not restimulated after 7 days of differentiation (**Figure 3.2**), suggesting Th1 cells need restimulation to drive maximal *Ifng* production.

In Th2 cells, *Il10* mRNA expression gradually increased from 0 to 6 hours post restimulation, at which point it peaked. Over the next four hours, from 6 to 10 hours post restimulation, *Il10* expression decreased to levels similar to those seen in Th1 cells, where it was maintained for the remainder of the time-course. The expression of *Il4* mRNA peaked at 6 hours post restimulation. *Il4* mRNA levels then decreased and were maintained at about half the maximal amount for the remainder of the time-course (**Figure 3.6**). Therefore, for differentiated Th1 and Th2 cells, *Il10* mRNA expression peaked between 4 to 6 hours, *Il2* expression peaked at 4 hours, and the hallmark cytokines peaked at 6 hours post restimulation.

3.4 Discussion

The anti-inflammatory roles of IL-10 are fundamental in regulating and maintaining a balanced immune response (Moore et al., 2001). CD4+ T cells are thought to be one of the major sources of IL-10 (Roers et al., 2004). However, owing to the complex nature of Th cell *in vitro* differentiation, a full understanding of the factors regulating IL-10 expression is incomplete. To further our understanding of IL-10 regulation in Th cells, we first needed to optimise the differentiation of different Th subsets *in vitro*, for both hallmark cytokine production and IL-10 production at the protein and mRNA level. Therefore, with the aim of finding the optimal *in vitro* differentiation conditions for Th1, Th2 and Th17 cells, we set out to test the effect of different culture conditions including TCR stimuli, culture durations, restimulation kinetics, and cytokine stimuli.

It is important to note that CD4+ T cells differentiated *in vitro* may not always be comparable to those that occur *in vivo* (Zhu et al., 2010). The study of *in vivo* specification of Th cells is important for understanding the factors involved in controlling infection, and crucial for identifying potential therapeutic interventions. It has been suggested that Th cells differentiated *in vitro* may represent the highly polarised cells that are found in unusual cases *in vivo*, such as during chronically infected or diseased tissue (Messi et al., 2003; O'Garra et al., 2011; Zhu et al., 2010). Nevertheless, the reductionist approaches of studying Th cells *in vitro* are central for investigating cells that are typically found in low numbers *in vivo*, and for in-depth molecular analysis of cytokine gene expression and regulation.

3.4.1 Different durations of differentiation are required for optimal hallmark cytokine and IL-10 production by different Th cell subsets

3.4.1.1 Robust Th1 and Th2 cell populations can be differentiated *in vitro* after 7 days

Analysis of Th1 and Th2 hallmark cytokines, IFNγ and IL-4, respectively, at the intracellular protein level revealed that the highest concentrations were produced after 7 days of culture. Moreover, IL-10 in Th1 and Th2 cells, at the intracellular protein and

mRNA level, peaked at day 7. Th2 cell IL-4 mRNA peaked at day 5 which fits with the fact that mRNA is translated into protein and therefore there is likely to be a delay in the detectability of protein. Furthermore, GATA3 in Th2 cells increased over the 7-day period. Th1 cell IFNγ mRNA peaked at day 3 and then decreased again, which did not reflect the intracellular protein profile of IFNγ, which peaked at day 5. This is not surprising, however, as it has been shown that Th cells often need restimulation to maintain high levels of cytokines, and that after initial stimulation cytokine expression is not maximal (O'Garra et al., 2011; Saraiva et al., 2009; Zeng, 2013). In these experiments, samples taken for mRNA analysis were not restimulated, whereas samples taken for intracellular protein analysis were restimulated. T-bet, the Th1 master regulator, increased over the 7 day period to a peak at day 7. Therefore, for hallmark cytokine and IL-10 production by Th1 and Th2 cells, 7 days is the optimal duration of culture.

The fact that IL-10 expression in Th1 and Th2 cells follows a similar expression pattern to the hallmark cytokines is not unexpected, as the factors that drive IL-10 in these cells are intertwined with those coordinating differentiation. In Th2 cells IL-4, STAT6 and GATA3 regulate IL-10 expression (Saraiva and O'Garra, 2010), and IL-10 is expressed constitutively. In Th1 cells IL-12, alongside strong TCR triggering, are required for maximal IL-10 expression (Saraiva et al., 2009). Furthermore, in Th1 cells IL-10 is thought to be expressed as the cells terminally differentiate, acting as negative feedback on APCs to control their IL-12 and proinflammatory cytokine production (O'Garra and Vieira, 2007; Trinchieri, 2007), and potentially causing Th1 anergy (O'Garra et al., 2011; Saraiva et al., 2009).

We also compared the cytokine expression profiles of differentiated Th1 and Th2 cells to *in vitro* cultured 'naïve' cells. Analysis of mRNA, protein and intracellular cytokine production of naive activated, Th0, Th1 and Th2 cells showed that we can differentiate naïve T cells into these fours subsets and they have the characteristics one would expect; naive activated and Th0 cells did not produce meaningful amounts of any cytokines other than IL-2; Th1 cells produced high levels of IFNγ and intermediate amounts of IL-10; Th2 cells produce high levels of IL-4 and IL-10. The differentiation of Th cells under these optimised conditions provided good representations and controls for subsequent studies. We have shown that, in accordance with previous publications

(Saraiva et al., 2009; Shoemaker et al., 2006), different T helper cell subsets have distinct cytokine profiles, and that they are in agreement with the proposed roles for these cells within the immune response. Th1 cells produce IFNγ to aid clearing of intracellular pathogens (O'Garra, 1998), while Th2 cells produce IL-4 to promote the removal of helminths (Zhu, 2010), they both produce IL-10 to downregulate excessive pathological responses (Jankovic et al., 2010; Saraiva and O'Garra, 2010).

Th0 cells are an *in vitro* subset driven in conditions designed to maintain them in an active naïve state. When Th cells are initially activated, before they encounter polarising cytokines, they produce IL-2 to drive their own proliferation but as they have not yet differentiated they do not express hallmark cytokines. Naive activated cells are also an *in vitro* subset, however unlike Th0 cells they are not suppressed with antibodies, and unlike Th1 and Th2 cells they are not driven in the presence of polarising cytokines. Therefore their lack of production of hallmark cytokines indicates that our conditions did not drive Th cells to differentiate into a specific subset unless polarising cytokines were added. Their lack of differentiation also indicates that we are stimulating with an optimal levels of anti-CD3 and anti-CD28, as work from our lab reveals that when naive activated cells are stimulated with too high or too low a dose they may spontaneously differentiate down a Th1 or Th2 like pathway, respectively (Saraiva et al., 2009). In addition, if the stimulation dose is too low naive activated cells may, under certain conditions, express FoxP3 (Gabrysova et al., 2011), which was not detectable in our naive activated population (data not shown). Therefore we are confident that our *in vitro* cultured Th1 or Th2 cells differentiated from naïve cells and have robust cytokine expression phenotypes that are distinct from each other.

3.4.1.2 Th17 cells produce optimal IL-10 after 3 days and optimal IL-17 after 5 days of differentiation *in vitro*

Our study of the Th17 cell hallmark cytokine, IL-17, at the intracellular protein and mRNA levels revealed that the largest amounts were produced after 5 days of culture. Furthermore, RORγt expression increased over the time-course to a plateau at day 3. However, IL-10 did not follow the same profile as IL-17. IL-10 mRNA expression peaked at day 2 and intracellular IL-10 protein peaked at day 3. By day 5, IL-10 mRNA and intracellular protein levels were reduced to less than those seen after 1 day of

differentiation. Therefore the optimal duration for culture of Th17 cells is complex as IL-17 and IL-10 peak on different days. Unlike IL-10 production by Th1 and Th2 cells, in Th17 cells IL-10 production is highly variable and inconsistent. This is likely to be due to the fact that Th17 cells are highly plastic, and it has been suggested they are constantly balancing between becoming 'pathogenic' or 'regulatory' (Bettelli et al., 2006). Pathogenic Th17 cells, which are promoted by IL-23 and IL-1β, begin to express IFNγ; regulatory Th17 cells, which are promoted by TGFβ, lose IL-17 expression and can begin to express FoxP3 and/or IL-10 (Beriou et al., 2009; Bettelli et al., 2006; Gagliani et al., 2015; Ghoreschi et al., 2010; Hirota et al., 2011; McGeachy et al., 2007; Zhou et al., 2008; Zielinski et al., 2012). Nevertheless, after 3 days of culture we managed to differentiate IL-17 and IL-10 producing Th17 cells that did not express IFNγ or FoxP3. Though at day 3 IL-17 expression was not at its peak, it was robust at both the mRNA and intracellular protein level, and RORγt expression had plateaued. Therefore we considered these cells to be differentiated Th17 cells that have not yet turned off IL-10 expression.

3.4.2 Th1, Th2 and Th17 cells are heterogeneous with regard to hallmark cytokine and IL-10 production

Intracellular cytokine staining of the hallmark cytokines and IL-10 in Th1, Th2 and Th17 cells revealed that cytokine production is highly heterogeneous within these populations of cells (**Figure 3.7**). Within the Th1 subset you have three distinct cytokine producing subpopulations: IFNγ- IL-10-, IFNγ+ IL-10- and IFNγ+ IL-10+. Within the Th2 subset you have four distinct cytokine producing subpopulations: IL-4- IL-10-, IL-4+ IL-10-, IL-4- IL-10+ and IL-4+ IL-10+. Within the Th17 subset you also have four distinct cytokine producing subpopulations: IL-17- IL-10-, IL-17+ IL-10-, IL-17- IL-10+ and IL-17+ IL-10+.

Interestingly, in the Th1 subset only the cells that produceed high levels of IFNγ also produceed IL-10. None of the Th1 cells that were negative for IFNγ produced IL-10, in contrast to what was seen for Th2 and Th17 cells, where IL-4 or IL-17 negative cells could make IL-10. This phenomenon supports concepts found in the literature. Highly active Th1 cells express IL-10 to downregulate themselves and reduce their

pathogenicity via an intercellular, paracrine feedback mechanism (O'Garra and Vieira, 2007; Trinchieri, 2007). APCs produce IL-12 in response to pathogens, which initiates the differentiation of Th1 cells that promote eradication of the pathogen (Hsieh et al., 1993b). However, to control this process the Th1 cells also make IL-10, which then suppresses the APC IL-12 production to reduce the Th1 response (Murray, 2006b). Th2 cells and Th17 cells, conversely, both produce their own driving cytokines, IL-4 and IL-6/TGFβ respectively (Fiorentino et al., 1989; Gutcher et al., 2011; Korn et al., 2009; Mosmann et al., 1986), and therefore this intercellular, paracrine feedback mechanism does not exist. However, the IL-10 Th2 and Th17 cells produce may feedback on the cells themselves in an autocrine manner to prevent IL-4 and IL-6/TGFβ production (Coomes, In Press. ; Huber et al., 2011; Murai et al., 2009). This may explain why we can see Th2 and Th17 cells that only produce IL-10 without their hallmark cytokine.

3.4.3 4 hours of restimulation is optimal for production of IL-10 and hallmark cytokines by Th cells

We have observed, and previous publications concur, that Th cells require restimulation to produce maximal amounts of cytokines (Helmstetter et al., 2015; O'Garra et al., 2011; Prussin and Metcalfe, 1995; Saraiva et al., 2009). Therefore we set out to investigate the best timings for restimulation of differentiated Th cells for mRNA expression of hallmark cytokines and importantly IL-10.

To address this we performed a time-course of restimulation of Th1 and Th2 cell cytokine mRNA expression. This analysis was not performed on Th17 cells, however, a microarray analysis run of mRNA kinetics from Th1, Th2 and Th17 cells (Gabrysova, Unpublished) found similar Th17 hallmark cytokine and *Il10* kinetics as those seen for Th1 and Th2 cells. mRNA levels for *Il10* and hallmark cytokines mostly peaked at 6 hours post restimulation. After 6 hours, *Il10*, *Ifng* and *Il4* levels dramatically decreased. This lead to the concern that mRNA levels may begin to decrease before cells could be fixed or lysed, as the process of removing cells from the culture plates and cell surface protein staining takes a considerable amount of time. Therefore, for future experiments, we decided to take samples of cells that had been restimulated for 4 hours.

3.4.4 Conclusions and future plans for studying the regulation of IL-10 in *in vitro* differentiated Th1, Th2 and Th17 cells

We have optimised the conditions in which Th1, Th2 and Th17 cells can be differentiated *in vitro* for hallmark cytokine and IL-10 production. Th1 and Th2 cells are cultured for 7 days, while Th17 cells are cultured for 3 days. All cells are restimulated for 4 hours after differentiation, before samples are taken. With these protocols in place, we can study the regulation of IL-10 within these Th cell subsets. However, as highlighted in **Figure 3.7**, these subsets are highly heterogeneous with regard to cytokine production. All of the Th cell subsets contain subpopulations of cells that express different combinations of cytokines. Therefore to fully elucidate what factors may be involved in the regulation of IL-10 in Th cells, in the following chapters, we have developed methods to separate these subpopulations and investigate the different molecular pathways that are present in each.

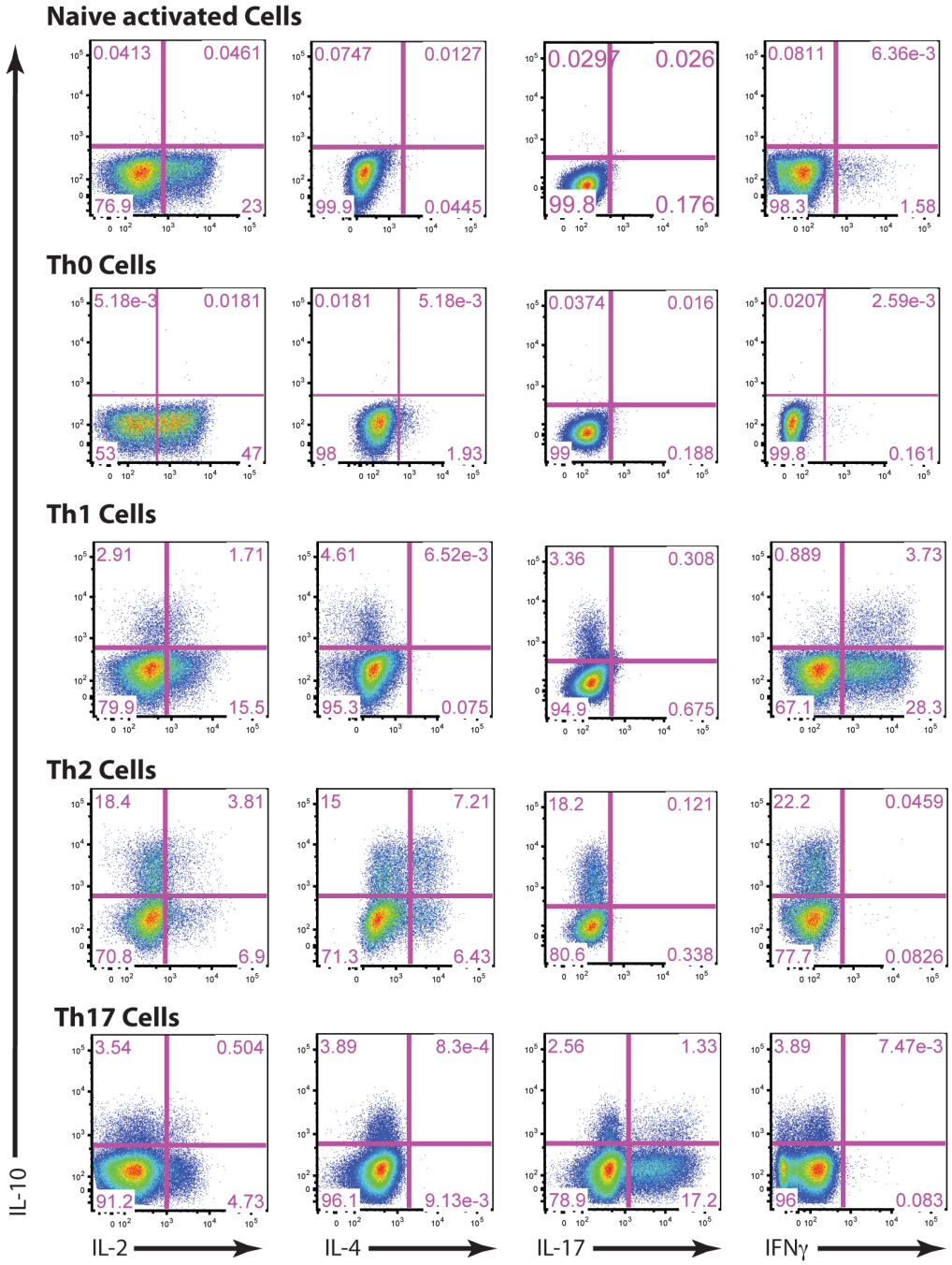


Figure 3.1 Intracellular IL-10 and hallmark cytokine production by Th1, Th2 and Th17 cells driven with anti-CD3 and anti-CD28 at different days after differentiation from naïve cells

C57BL/6 naive CD4+CD62L+CD44loCD25- cells were driven with anti-CD3 and anti-CD28, and polarised to Th1 cells with IL-12 and anti-IL-4 or Th2 cells with IL-4, IL-2, anti-IL-12 and anti-IFNγ or Th17 cells with TGFβ, IL-6, anti-IL-4, anti-IL-12 and anti-IFNγ. Plots of flow cytometric analysis of intracellular cytokine staining, cells were assessed after 1, 2, 3, 5 or 7 days of polarization *in vitro* and restimulated for ICS as described in Materials and Methods. Numbers show percentage of live CD4+ cells. Representative of one experiment.

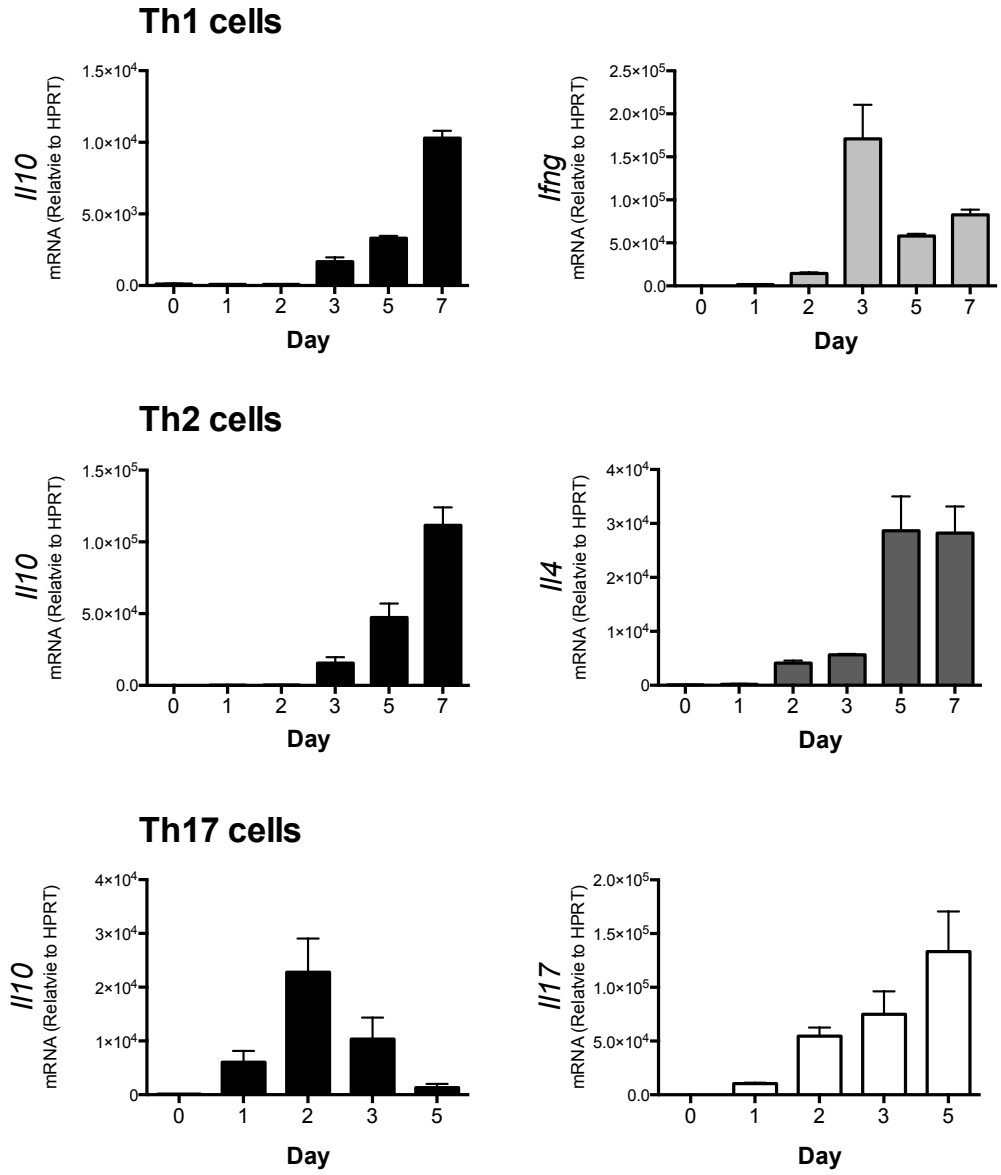


Figure 3.2 mRNA of cytokine production by Th1, Th2 and Th17 cells driven with anti-CD3 and anti-CD28 at different days after differentiation from naïve cells

C57BL/6 naive CD4+CD62L+CD44loCD25- cells were driven with anti-CD3 and anti-CD28, and polarised to Th1 cells with IL-12 and anti-IL-4 or Th2 cells with IL-4, IL-2, anti-IL-12 and anti-IFNγ or Th17 cells with TGFβ, IL-6, anti-IL-4, anti-IL-12 and anti-IFNγ. Graphs of transcription factor mRNA measured by qPCR relative to HPRT. Cells were harvested and mRNA was extracted with no restimulation after 1, 2, 3, 5 or 7 days of polarization *in vitro*. Graphs show means ± SD of triplicates. Representative of one experiment.

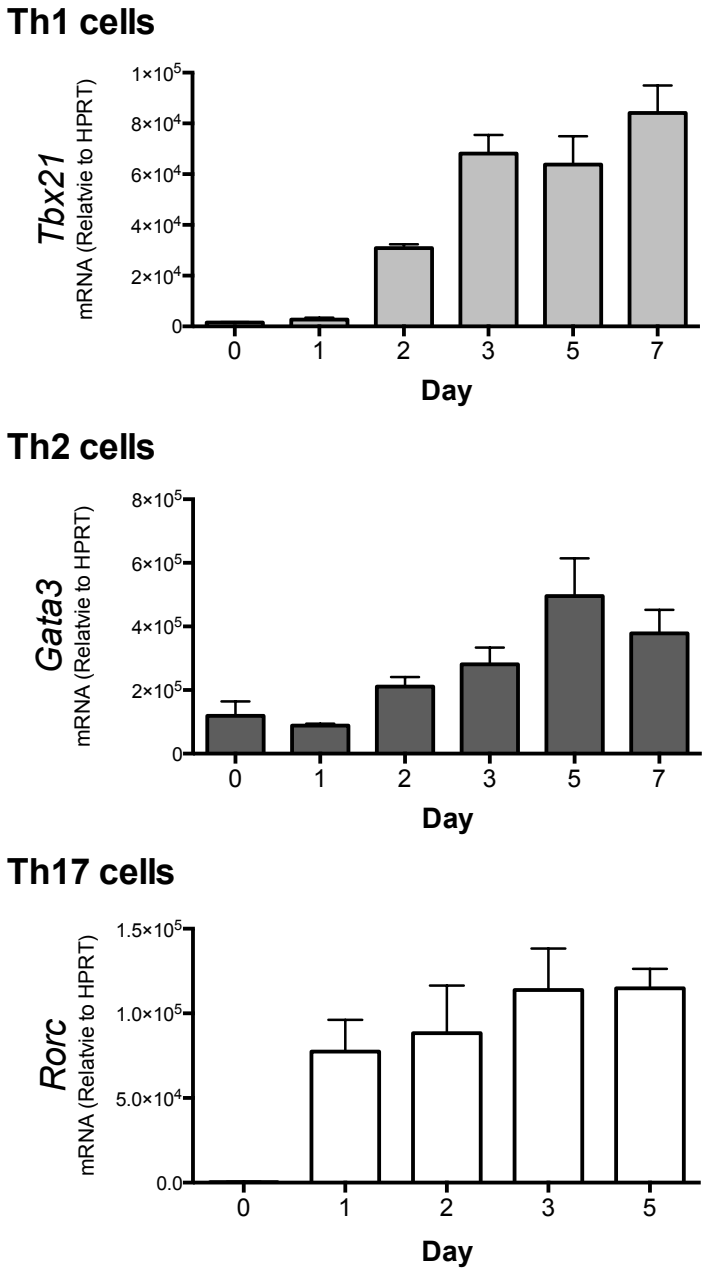


Figure 3.3 mRNA of master transcription factor expression by Th1, Th2 and Th17 cells driven with anti-CD3 and anti-CD28 at different days after differentiation from naïve cells

C57BL/6 naive CD4+CD62L+CD44loCD25- cells were driven with anti-CD3 and anti-CD28, and polarised to Th1 cells with IL-12 and anti-IL-4 or Th2 cells with IL-4, IL-2, anti-IL-12 and anti-IFNγ or Th17 cells with TGFβ, IL-6, anti-IL-4, anti-IL-12 and anti-IFNγ. Graphs of transcription factor mRNA measured by qPCR relative to HPRT. Cells were harvested and mRNA was extracted with no restimulation after 1, 2, 3, 5 or 7 days of polarization *in vitro*. Graphs show means ± SD of triplicates. Representative of one experiment.

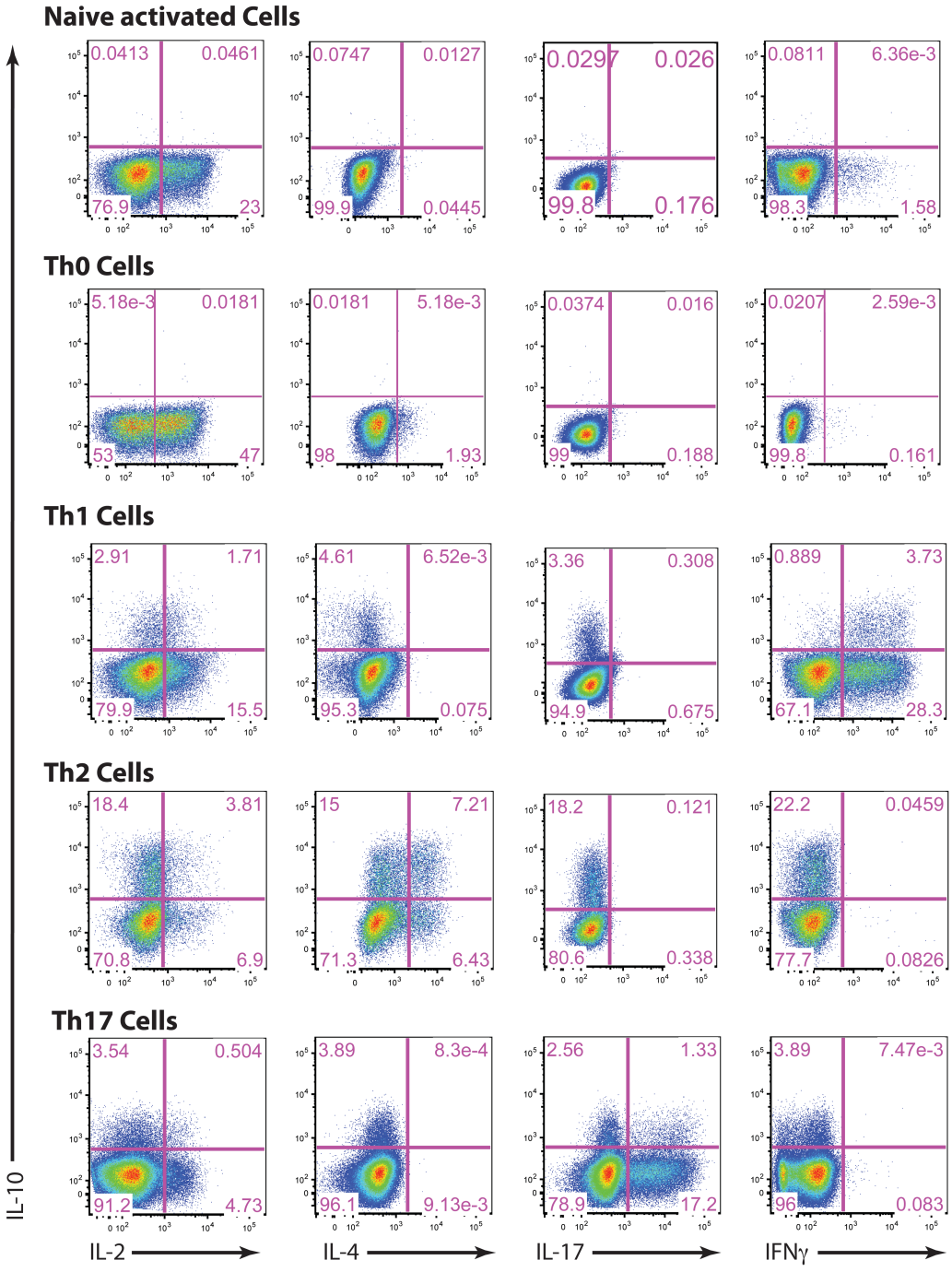


Figure 3.4 Intracellular cytokine production by naïve activated, Th0, Th1, Th2 and Th17 cells after optimal days of differentiation for peak cytokine production

C57BL/6 naive CD4+CD62L+CD44loCD25- cells were driven with plate-bound anti-CD3 and soluble anti-CD28, and polarised to naïve activated cells with medium alone; Th0 cells with anti-IL-4, anti-IL-12, anti-IFNγ, anti-IL-6 and anti-TGFβ; Th1 cells with IL-12 and anti-IL-4; Th2 cells with IL-4, IL-2, anti-IL-12 and anti-IFNγ; Th17 cells with TGFβ, IL-6, anti-IL-4, anti-IL-12 and anti-IFNγ. Plots of flow cytometric analysis of intracellular cytokine staining, naïve activated, Th0, Th1 and Th2 cells were assessed after 7 days of polarization *in vitro,* Th17 cells were assessed after 3 days of polarization *in vitro*. Cells were restimulated for ICS as described in Materials and Methods. Numbers show percentage of live CD4+ cells. Representative of two to three experiments.

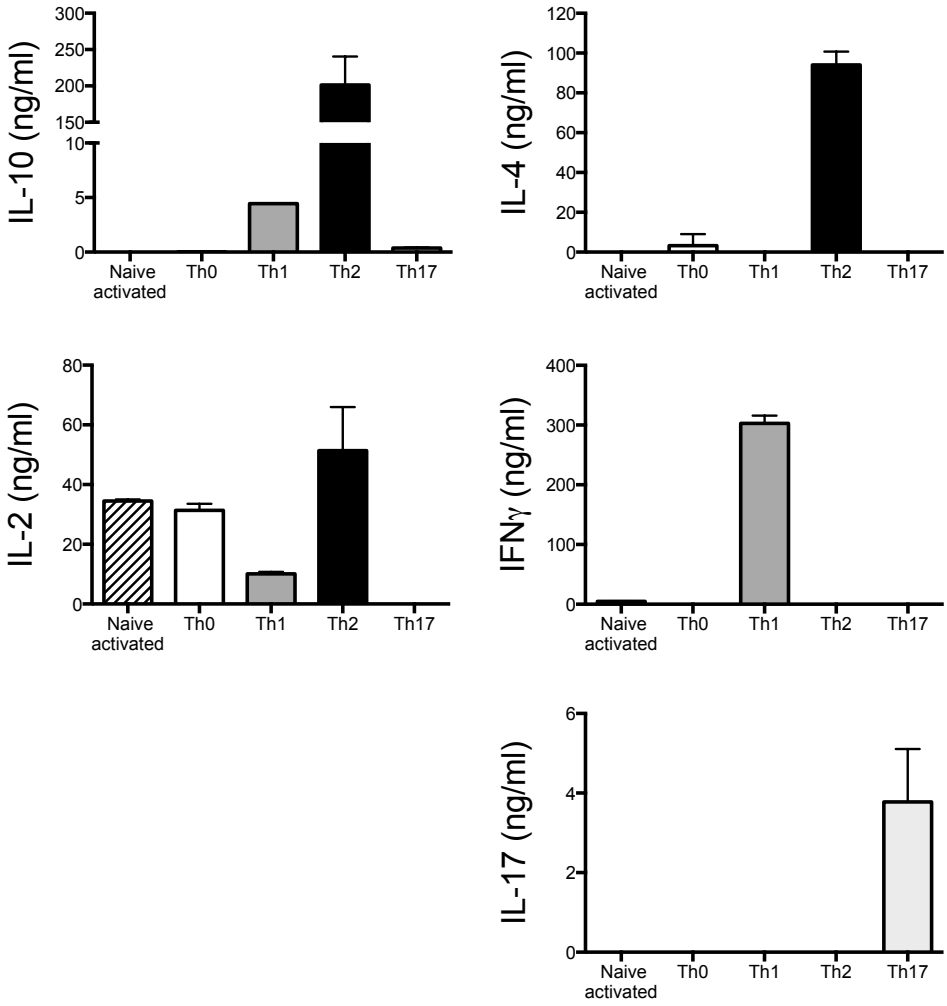


Figure 3.5 ELISA of IL-10 and hallmark cytokine protein production by naïve activated, Th0, Th1, Th2 and Th17 cells after optimal days of differentiation

C57BL/6 naive CD4+CD62L+CD44loCD25- cells were driven with plate-bound anti-CD3 and soluble anti-CD28, and polarised. Naïve activated cells with medium alone; Th0 cells with anti-IL-4, anti-IL-12, anti-IFNg, anti-IL-6 and anti-TGFb; Th1 cells with IL-12 and anti-IL-4; Th2 cells with IL-4, IL-2, anti-IL-12 and anti-IFNγ; Th17 cells with TGFβ, IL-6, anti-IL-4, anti-IL-12 and anti-IFNγ. Graphs of enzyme-linked immunosorbent assay of cytokines in supernatants. Naïve activated, Th0, Th1 and Th2 cells were assessed after 7 days of polarization in vitro, Th17 cells were assessed after 3 days of polarization in vitro. Cells were restimulated for ELISA as described in Materials and Methods. Graphs show means ± SD of triplicates. Representative of thwo experiments.

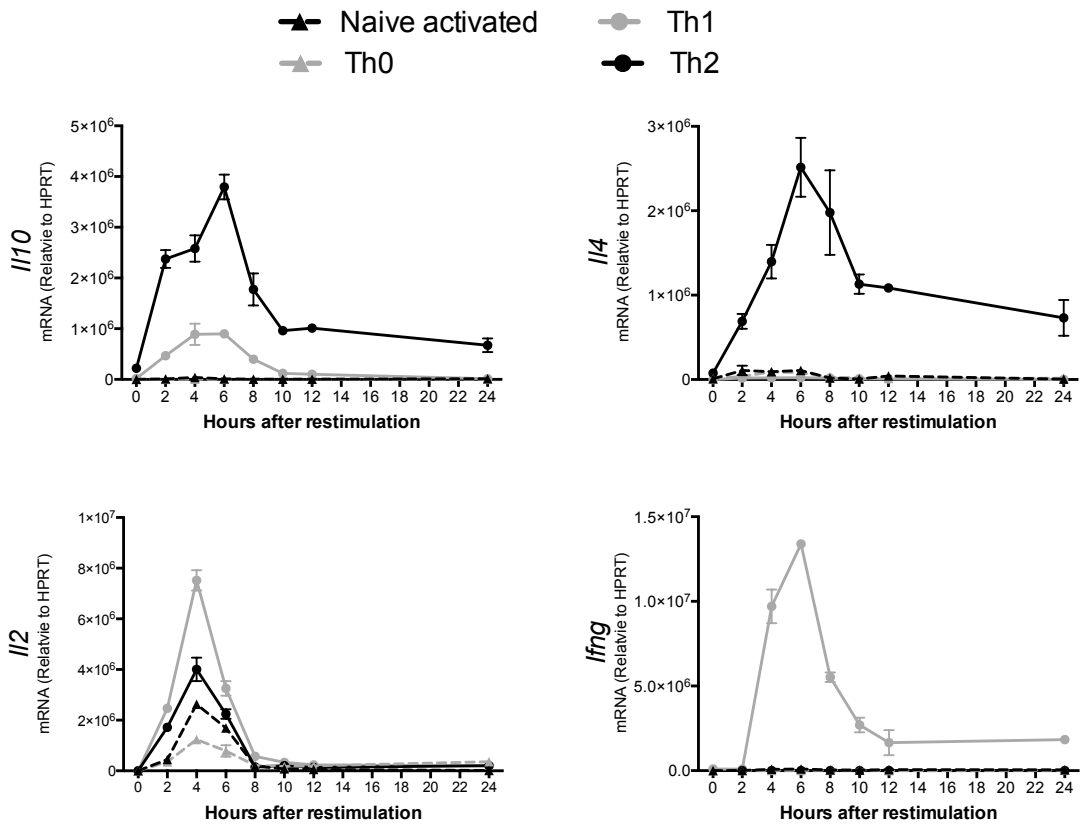


Figure 3.6 mRNA of cytokines expressed upon restimulation of naïve activated, Th0, Th1 and Th2 cells driven with anti-CD3 and anti-CD28 after 7 days of differentiation from naïve cells

C57BL/6 naive CD4+CD62L+CD44loCD25- cells were driven with plate-bound anti-CD3 and soluble anti-CD28, and polarised to NIL cells with medium alone, Th0 cells with anti-IL-4, anti-IL-12, anti-IFNγ, anti-IL-6 and anti-TGFβ, Th2 cells with IL-4, IL-2, anti-IL-12 and anti-IFNγ or Th1 cells with IL-12 and anti-IL-4. Graphs of cytokine mRNA measured by qPCR relative to HPRT. Cells were harvested after 7 days of polarization *in vitro* and restimulated for the specified time with plate-bound anti-CD3 and soluble anti-CD28. Cells were restimulated for mRNA extraction as described in Materials and Methods. Representative of one to two experiments.

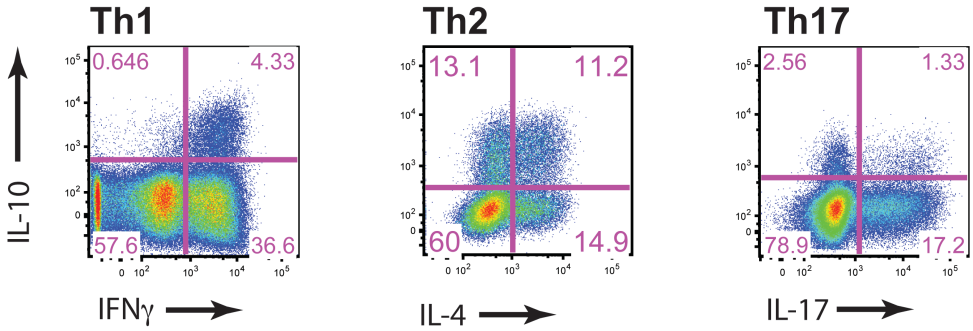


Figure 3.7 Summary of the heterogeneity in IL-10 and hallmark cytokine production by Th1, Th2 and Th17 cells when differentiated after optimal days of differentiation

C57BL/6 naive CD4+CD62L+CD44loCD25- cells were driven with plate-bound anti-CD3 and soluble anti-CD28, and polarised to Th2 cells with IL-4, IL-2, anti-IL-12 and anti-IFNγ, or Th1 cells with IL-12 and anti-IL-4, or Th17 cells with TGFb, IL-6, anti-IL-4, anti-IL-12 and anti-IFNγ. Plots of flow cytometric analysis of intracellular cytokine staining, cells restimulated as described in Materials and Methods, 3 days for Th17 cells and 7 days for Th1 and Th2 cells of polarization in vitro. Numbers show percentage of live CD4+ cells. Representative of three to six experiments.

Chapter 4. The optimisation of RNA extraction and RNA-Sequencing analysis of intracellular cytokine stained T helper cell subsets

4.1 Background

CD4+ T cells are fundamental to a balanced and effective immune response. The different Th subsets secrete particular proinflammatory cytokines that drive specific immune responses to eradicate the invading pathogens (Zhu et al., 2010). However, these proinflammatory actions can also be harmful to the host if excessive and unregulated or inappropriate. Therefore, Th cells also secrete the regulatory cytokine IL-10 (Maynard and Weaver, 2008; Saraiva and O'Garra, 2010), that feeds back directly and indirectly to reduce CD4+ T cell proinflammatory cytokine secretion and proliferation (Ouyang et al., 2011). Each Th subset secretes a repertoire of different cytokines, but as shown in Chapter 3 (**Figure 3.7**), within that Th population the cells have heterogeneous cytokine secretion profiles. It is increasingly understood that genes associated with subpopulations of cells within a heterogeneous population cannot be revealed unless these cells are separated from the bulk population (Mahata et al., 2014) (Kuchroo VK, International Congress of Immunology, 2013). Therefore, to better understand the common and disparate mechanisms involved in regulating hallmark cytokine and IL-10 expression, we want to separate the Th subsets into subpopulations and analyse the differential gene expression patterns within these populations using RNA-Seq. By doing this we hope to glean information from different cytokine secreting subpopulations, particularly IL-10 producers, which could be difficult to detect when analysing only the bulk population of a Th subset.

Four approaches could be used to separate these Th cell subpopulations: 1) using a cytokine secretion assay, 2) using transgenically modified cytokine reporter mice, 3) using single cell RNA-Seq, 4) using intracellular cytokine staining (ICS). These three approaches all have pros and cons, which I will now discuss.

Cytokine secretion assays can be used to detect cytokines secreted by cells. With these assays, cells are labelled with cytokine specific ‘catch-reagents’ that then bind the specific cytokine when it is secreted by that cell. These cytokines are subsequently labelled with specific detection fluorescent antibodies, which can then be detected by FACS (Kaufmann and Kabelitz, 2002). Unlike ICS, cells selected with the cytokine secretion assay can continued to be cultured (Chang et al., 2007) or used for other downstream analysis, such as the analysis of RNA (Ahyi et al., 2009). However, these assays do have disadvantages, for example not all secreted cytokine is caught and

therefore production can be underrepresented (Helmstetter et al., 2015; Kaufmann and Kabelitz, 2002). Furthermore, diffuse cytokine molecules accumulate in the culture and other cells can catch low amounts of the cytokine and are subsequently labelled (Kaufmann and Kabelitz, 2002); resulting in non-cytokine producing cells being labelled as cytokine producing. This technique is further limited by the availability of specific cytokine assays, which are only produced for a small selection of cytokines in a limited selection of fluorochromes.

In the last 15 years multiple mouse strains have been developed that are transgenically modified to express a reporter, such as a fluorescent molecule, whenever a gene is expressed. For example the '4get' mouse, where green fluorescent protein (GFP)(Tsien, 1998) has been incorporated into the *Il4* locus such that a fluorochrome molecule is expressed whenever the IL-4 gene is transcribed (Mohrs et al., 2001). However, one can only look at one specific cytokine with these reporter mice. Therefore, dual reporter mice have been developed that can report on two cytokines at once. For example the '4get' mouse, reporting IL-4 as GFP, has been crossed to the 'YETI' mouse, where yellow fluorescent protein (YFP) has been incorporated into the *Ifng* locus (Stetson et al., 2003). Thus these mice can report IL-4 (GFP) and IFNγ (YFP) simultaneously. The major benefit of this system is that using this mouse one can track cells that are expressing certain cytokines without having to disrupt the cells with techniques such as fixation or permeablisation that may reduce mRNA quality. However, there are several caveats to this system. Firstly, reporter mice have only been developed for a handful of cytokines, and therefore we would have to generate new reporter mice for any cytokines that do not currently have corresponding reporter mice. Secondly, as we want to look at IL-10 alongside the hallmark cytokines we would have to cross multiple reporter mice strains. Furthermore, if in the future we want to look at the cytokine secreting Th cell subpopulations in gene knockout mice we would have to again cross our reporter mice strains with these knockout stains, and this method could never be used on human cells. All of which would be very costly and time-consuming. Simultaneously, another facet of separating Th cells based on this method is that fluorescent reporters do not fully correlate to intracellular protein production (Kamanaka et al., 2006; Mohrs et al., 2005). The fluorescent reporter is transcribed alongside the cytokine's mRNA, however, as previously discussed, cytokines have many checkpoints of post-transcriptional regulation before they are translated into protein. Therefore there is a risk that the

fluorescence is reporting all cells that are transcribing the cytokine, some of which may not produce the protein.

Single cell RNA-Seq is a technique by which individual cells from within a heterogeneous population of cells can be analysed at the mRNA level (Tang et al., 2010a; Tang et al., 2010b; Tang et al., 2009). Using this technique it is possible to differentiate Th cells and then process multiple individual cells from within the population for their mRNA expression profiles to establish what cytokines they were expressing, and what genes are associated with these expression profiles. This can be performed on cells from any organism including humans and knockout mice, and the cells are not disrupted in any way prior to mRNA extraction. However, there are many limitations at both the processing and analysis stages with this technique (Saliba et al., 2014; Tang et al., 2011). As this technique selects individual cells, there is often a bias towards more highly expressed genes in the processing of the samples with low-expression genes showing higher inter-sample variability (Wu et al., 2014). Furthermore, the selection of cells taken for the single cell RNA-Seq is effectively random and therefore many samples are required for representative coverage – there is always a risk that certain cytokine expressing subpopulations will not be selected. Finally, and primarily for our study, we want to be able to perform a comparison between protein production and mRNA expression. Single cell RNA-Seq does not allow us to do this as it only assesses cells at the mRNA level, and as we are aware cells can express high levels of a certain mRNA without actually producing detectable amounts of the protein (Powell et al., 2000), this technique is not well suited for this project.

We want to tie IL-10 and hallmark cytokine protein production to mRNA expression, and to transcription factors and other genes associated with *Il10* regulation, and we want to be able to do this with cells from any mouse stain or from humans. Therefore, we decided to investigate if we could use ICS and fluorescence assisted cell sorting (FACS) to separate Th cell subpopulations for RNA-Sea analysis. ICS and flow cytometric techniques for analysis of CD4+ T cells are well developed within the O'Garra lab (Barrat et al., 2002; Gabrysova et al., 2011; Murphy et al., 1996; Openshaw et al., 1995; Saraiva et al., 2009; Vieira et al., 2004). However a major obstacle is that for ICS the cells must be fixed and permeablised, to enable the cytokine staining antibodies to enter the cells. Once fixed and permeablised there is very little further analysis one can perform on them since the cells are dead and a major concern with performing FACS on

intracellular stained samples is that fixation is known to fragment mRNA (Opitz et al., 2010), and this may hinder RNA-Seq analysis. However, there is literature suggesting that microarray can be performed on fragmented mRNA (Haller et al., 2006; Hodgin et al., 2010), but it may be a concern for RNA-Seq analysis. To overcome this a recent publication implemented a technique that enables RNA-Seq analysis of different cell subpopulations based on protein as seen by intracellular protein staining by incorporating RNase inhibitors throughout the protocol (Hrvatin et al., 2014a). Though this technique was developed on human stem cells, and was not performed on cytokines, practices of this protocol could be used to aid us in developing a method for separating Th cell subset subpopulations based on their cytokine protein profiles for analysis by RNA-Seq.

4.2 Separating T helper cells into different cytokine expressing populations for analysis by RNA-Seq: Study Aims

We want to establish a method for separating Th cell subset subpopulations based on their intracellular cytokine protein profiles for analysis by RNA-Seq. However, when starting this project there was no literature on performing RNA-Seq on ICS samples and fixation is thought to fragment mRNA. Therefore we set out to fulfil the following aims, with the overall aim of designing an optimised protocol for performing RNA-Seq on ICS samples.

1. Establish a protocol in which mRNA, which is suitable for RNA-Seq analysis, can be extracted from ICS samples.
2. Establish if mRNA from ICS samples is comparable to mRNA from non-ICS samples.
3. To validate this technique: when performing RNA-Seq on ICS Th1 and Th2 cells, are the gene expression profiles as expected from the literature?

4.3 Results

4.3.1 Investigating the quality of mRNA extracted from intracellular cytokine stained samples

We were interested in separating Th cell subpopulations based on their intracellular cytokine profiles for analysis by RNA-Seq. However, the literature suggested that standard the protocol for ICS (**Figure 4.1 A**) may result in low quality degraded mRNA (Opitz et al., 2010). Cells prepared for ICS (**Figure 4.1 A**) or mRNA extraction (**Figure 4.1 B**) are both restimulated after *in vitro* differentiation, however after this step bulk populations of cells for mRNA are normally immediately lysed and frozen, while cells for ICS are stained, fixed and permeablised. We wanted to find a way to combine these two protocols; to isolate mRNA from samples after flow cytometric sorting of the Th cell subpopulations. From now on mRNA isolated from ICS samples (**Figure 4.1 A**) will be referred to as 'fixed' while mRNA isolated using the standard mRNA preparation protocol (**Figure 4.1 B**) will be referred to as 'unfixed'. Therefore we set out to investigate the quality of mRNA extracted from ICS 'fixed' samples to ascertain whether it could be used for RNA-Seq analysis.

4.3.1.1 mRNA from fixed samples is comparable to mRNA from unfixed samples when analysed by qPCR

Th1 and Th2 cells were cultured *in vitro* as described in Chapter 3. These samples were then split in half, with one portion being prepared for ICS (**Figure 4.1 A**) and, as a comparison, the other portion being prepared for standard mRNA extraction (**Figure 4.1 B**). mRNA from these samples was analysed by qPCR. We found that the fixed and unfixed Th1 samples had equivalent levels of *Ifng* mRNA and undetectable amounts of *Il4* mRNA (**Figure 4.2 A**). Fixed Th2 cells were shown to express slightly more *Il4* mRNA than unfixed Th2 cells. Both fixed and unfixed Th2 samples expressed undetectable amounts of *Ifng* mRNA (**Figure 4.2 A**). Therefore mRNA from fixed Th samples could be analysed by qPCR and the levels of hallmark cytokine mRNA were comparable to unfixed samples.

4.3.1.2 mRNA from fixed samples is of low quality

Th1 and Th2 cells were cultured *in vitro* as described in Chapter 3. These samples were then split in half, with one portion being prepared for ICS (**Figure 4.1 A**) and the other portion being prepared for standard mRNA extraction (**Figure 4.1 B**). The integrity of the mRNA from these samples was analysed by electropherogram (**Figure 4.2 B**). mRNA from unfixed samples had high RNA integrity numbers (RINs), reflective of good RNA quality, with clear 18S and 28S rRNA peaks, the 28S peak being larger than the 18S peak and with little noise between the peaks or before the 18S peak. However, mRNA from the fixed samples had low RINs, having lost both peaks and there was increased noise throughout the electropherogram. Furthermore, there was significantly less material in the fixed samples (**Figure 4.2 B**). This showed mRNA from fixed samples is fragmented or degraded.

4.3.1.3 mRNA from fixed samples is degraded when cells are permeablised

Th2 cells were cultured *in vitro* as described in Chapter 3. These samples were then prepared for ICS (**Figure 4.1 A**), with samples for mRNA extraction being taken at each step of the ICS process. Samples were taken: 1) straight after restimulation, as with standard mRNA preparation, 2) after restimulation and formaldehyde fixation, 3) after restimulation, formaldehyde fixation and permeablisation, 4) after restimulation, formaldehyde fixation, permeablisation and intracellular cytokine staining. The integrity of these mRNA samples was analysed by electropherogram (**Figure 4.3 A**). mRNA from unfixed samples in condition 1) had high RINs, with clear 18S and 28S rRNA peaks and little noise between the peaks or before the 18S peak. The same was true for fixed samples that had not been permeablised in condition 2). However, after permeablisation, in conditions 3) and 4), the samples had low RINs, having lost both peaks and there was increased noise throughout the electropherogram (**Figure 4.3 A**). Having confirmed that permeablisation, rather than fixation, led to reduced RINs, we wanted to establish if the RNA was fragmented or degraded. If the samples were fragmented then RNA-Seq should be possible and previous publications have shown that microarray can be performed on fragmented samples (Haller et al., 2006; Hodgin et al., 2010). However, if the samples were degraded then information would be lost and RNA-Seq analysis would not be achievable. Therefore we performed RNA-Seq on

samples prepared in conditions 1) which were not fixed and had high RINs (referred to as unfixed), or 4) which were fixed, permeablised and stained and had low RINs (referred to a fixed). We wanted to establish if permeablisation led to alterations in the representation of individual transcripts by looking for bias in gene coverage (**Figure 4.3 B**). We found that fixed samples had an increased detection of transcripts at the 3' end relative to the 5' end, demonstrating a 3'-coverage bias (**Figure 4.3 B**). This is indicative of mRNA degradation by ribonucleases (Auer et al., 2003), rather than fragmentation.

4.3.2 Investigating whether the addition of RNase inhibitors prevents RNA degradation in fixed samples

Our data suggested that ribonucleases, also known as RNases, may have caused the mRNA degradation we saw upon permeablisation of samples. This is supported by a recent publication, which performed microarray and RNA-Seq analysis on fixed intracellular protein stained human cells, and showed that if the staining protocol was performed in the presence of high concentrations of RNase inhibitors, then high quality RNA can be extracted from the samples (Hrvatin et al., 2014a). Therefore we wanted to establish whether the RNase inhibitors would prevent the degradation of mRNA we see after preparing samples for ICS. To do this we added RNase inhibitors from the fixation step onwards (**Figure 4.4**). We wanted to ensure that mRNA extracted from these samples was of high quality and comparable to mRNA from unfixed samples, and without the loss of detection of particular transcripts. Therefore we set up a study in which Th1 and Th2 cells were differentiated *in vitro* and then the whole population of Th cells was split into two and restimulated, as optimised in Chapter 3 and described in Materials and Methods. One portion of the cells was immediately transferred into RLT buffer and RNA was extracted using the RNeasy Micro kit (**Figure 4.5 A**). The other portion of cells was prepared using the ICS protocol shown in **Figure 4.4** with the addition of RNase inhibitors, and cells were sorted on the flow cytometer for live CD4+ cells and RNA was extracted using the FFPE RNeasy kit (**Figure 4.5 A**).

The integrity of the mRNA extracted from these samples was analysed by electropherogram (**Figure 4.5 B**). mRNA from the unfixed Th1 and Th2 samples had high RINs of 10, with clear 18S and 28S rRNA peaks and little noise (**Figure 4.5 B**).

mRNA from the fixed Th1 and Th2 samples also had high RINs of 9, with clear rRNA peaks and little noise throughout the electropherogram (**Figure 4.5 B**). To further assess the quality of the samples we ran RNA-Seq on the RNA and assessed the representation of individual transcripts by looking for bias in the gene coverage (**Figure 4.5 C**). We found that though the fixed samples had a very slight increase in the detection of transcripts at the 3' end relative to the 5' end, it was not sufficient to be regarded as a 3'-coverage bias. In fact, there was a more even coverage of the gene bodies in the fixed samples, as the unfixed samples had a slight 5'-coverage bias (**Figure 4.5 C**). This indicates that the addition of RNase inhibitors throughout the ICS protocol prevents the degradation of mRNA from fixed samples, allowing for RNA-Seq analysis to fully cover the 3' to 5' of genes.

4.3.3 Investigating the similarity between fixed and unfixed Th1 and Th2 samples

4.3.3.1 Comparison of fixed and unfixed Th1 and Th2 samples at the whole gene expression level by RNA-Seq

Having established that we could extract high quality mRNA from ICS samples when in the presence of RNase inhibitors, we set out to further establish the similarities between the fixed and unfixed samples. We wanted to conclusively determine whether fixed samples were equivalent to unfixed samples. The samples were processed for RNA-Seq and analysed as described in the Materials and Methods.

The first test we ran to compare the unfixed and fixed samples involved calculating the Pearson's coefficient between the fixed and unfixed samples within each replicate experiment for Th1 cells and Th2 cells (**Figure 4.6**). In the coefficient, an R^2=1 means that the samples are identical. For each of the three experiments, comparing fixed and unfixed Th1 or Th2 cells, the R^2 was consistently greater than 0.96 (**Figure 4.6**). Therefore, the interpretation from the Pearson's Coefficients was that the fixed and unfixed samples were reliably almost indistinguishable. However, as the R^2 were not equal to 1, it was considered that some differences might exist between the samples. Therefore we further analysed these samples to try to establish what these differences were and whether they were introducing a significant bias to the RNA-Seq analysis.

For the Th1 cells we averaged the number of genes expressed in each of the six samples: three unfixed samples, and three fixed samples (11,848 genes). 93% of these genes were expressed in all six samples, both in the fixed and unfixed samples (**Figure 4.7 A**). Therefore, on average, 7% of genes did not overlap between the fixed and unfixed samples; 6% of these genes were expressed in only the fixed samples, and 1% of the genes were expressed in only the unfixed samples (**Figure 4.7 A**). It is possible that the ICS protocol may lead to the loss or overrepresentation of certain mRNA molecules. Consequently we looked at greater depth into whether these genes were consistently differentially expressed between the two conditions. Looking at each repeat individually, we compiled a list of the genes that were expressed in unfixed samples but not expressed in the fixed samples. For repeat 1, this list consisted of 101 genes, which was 0.9% of the total number of genes expressed by that sample (**Figure 4.7 B**). For repeat 2, there were 50 genes, which was 0.4% of the total, and for repeat 3 there were 274 genes, which was 2.3% of the total (**Figure 4.7 B**). Of these genes, only 27 were present in any two of the three repeats. None of the genes were present in all three of the repeats (**Figure 4.7 B**). Therefore, though there were some genes that were only expressed in the unfixed samples, no genes were repeatably overrepresented in the unfixed samples. Next, we compiled a list of the genes that were expressed in fixed samples but not expressed in the unfixed samples. For repeat 1, this list consisted of 1035 genes, which was 8.8% of the total number of genes expressed by that sample (**Figure 4.7 C**). For repeat 2, there were 684 genes, which was 5.7% of the total, and for repeat 3 there were 323 genes, which was 2.8% of the total (**Figure 4.7 C**). Of these genes, 344 were present in any two of the three repeats. 97 of the genes were present in all three of the repeats (**Figure 4.7 C**), which was 0.8% of the 11,848 average total number of genes expressed in the samples. Therefore, though there were some genes that were only expressed in the fixed samples, very few were repeatably overrepresented in the fixed samples, indicating these may be random differences.

We performed a similar analysis with the Th2 cells. For the Th2 cells we averaged the number of genes expressed in each of the six samples: three unfixed samples, and three fixed samples (12,357 genes). 92% of these genes were expressed in all six samples, both in the fixed and unfixed samples (**Figure 4.8 A**). Therefore, on average, 8% of genes did not overlap between the fixed and unfixed samples; 2% of these genes were expressed in only the fixed samples, and 6% of the genes were expressed in only the unfixed samples (**Figure 4.8 A**). Looking at each repeat individually, we compiled a list

of the genes that were expressed in unfixed samples but not expressed in the fixed samples. For repeat 1, this list consisted of 145 genes, which was 2.0% of the total number of genes expressed by that sample (**Figure 4.8 B**). For repeat 2, there were 400 genes, which was 3.4% of the total, and for repeat 3 there were 1408 genes, which was 10.7% of the total (**Figure 4.8 B**). Of these genes, only 203 were present in any two of the three repeats. 53 of the genes were present in all three of the repeats (**Figure 4.8 C**), which was 0.4% of the 12.357 average total number of genes expressed in the samples. We also compiled a list of the genes that were expressed in fixed samples but not expressed in the unfixed samples. For repeat 1, this list consisted of 364 genes, which was 3.0% of the total number of genes expressed by that sample (**Figure 4.8 C**). For repeat 2, there were 184 genes, which was 1.6% of the total, and for repeat 3 there were 134 genes, which was 1.0% of the total (**Figure 4.8 C**). Of these genes, 106 were present in any two of the three repeats. 13 of the genes were present in all three of the repeats (**Figure 4.8 C**), which was 0.1% of the 12,357 average total number of genes expressed in the samples. Therefore, though there were some genes that were only expressed in the fixed or unfixed samples, very few were repeatably overrepresented in the either condition.

To further establish the similarity between the fixed and unfixed samples we analysed the differential gene expression, pooling the results from the three repeats to enable statistical power. The magnitude of differential gene expression between fixed and unfixed Th1 cells and fixed and unfixed Th2 cells was analysed and represented as volcano plots (**Figure 4.9 A**). Each dot represents a gene transcript that was detectable in both the fixed and unfixed samples. Volcano plots report significance of differential expression (concentrated P value) as a function of fold change. Genes that were differentially expressed between fixed and unfixed samples at $P < 0.05$ are indicated by coloring. Blue indicates > 2.0 fold change in expression, red indicates < 2.0 fold change in expression (**Figure 4.9 A**). The actually number of differentially expressed genes at $P < 0.05$ and different fold changes for Th1 and Th2 cells are shown in **Figure 4.9 B**. In Th1 cells there were no significantly differentially expressed genes. Therefore, from a statistical standpoint the Th1 fixed and unfixed samples over three repeat experiments could be considered identical (**Figure 4.9 B**). In the Th2 cells, 21 genes (0.17% of total 12,652 genes) were significantly differentially expressed between the fixed and unfixed samples. At a cut-off of FC > 2.0, 8 genes (0.06% of total) were significantly differentially expressed between the fixed and unfixed samples (**Figure 4.9 B**).

Therefore, though there were 0.06% of the 12,652 genes that were significantly differentially expressed between Th2 fixed and unfixed samples, this was negligible in comparison to the similarities between the samples. Consequently, we concluded that the fixed and unfixed Th1 and Th2 samples were comparable at the RNA expression level.

4.3.3.2 Comparing fixed and unfixed Th1 and Th2 samples at the individual gene expression level

To further determine if the fixed and unfixed Th1 and Th2 samples are comparable, and that these samples are expressing the genes we would expect, we examined the gene expression profiles of the samples. To do this we chose to look at the relative expression of seven key genes that are known to be highly expressed by either Th1 or Th2 cells or both. The Th1 associated genes we chose were *Ifng* and *Tbx21*, the Th2 associated genes were *Il4, Il5, Il13* and *Gata3*. *Il10* was chosen as a gene that should be expressed in both subsets. The relative expression of these genes is shown as a bar graph (**Figure 4.10**). The error bars represent the relative expression across the three repeat experiments. The error bars were found to be almost negligible, supporting the previous result that there is little variation between the repeats. Furthermore, there was no significant difference between the fixed and unfixed samples for any of the genes analysed, further supporting that the fixed and unfixed samples, as prepared using novel protocols described in this chapter, were comparable. Finally, as expected the Th1 samples expressed considerably more *Ifng* and *Tbx21* than the Th2 cells, while the Th2 cells expressed considerably more *Il4, Il5* and *Il13* (**Figure 4.10**). Though *Gata3* was more highly expressed in the Th2 cells than the Th1 cells, this was not as noticeably different as it is for the other genes (**Figure 4.10**), which may be expected as discussed earlier GATA3 is required for CD4+ T cell development. *Il10* was expressed to almost equal amounts in both the Th1 and Th2 cells (**Figure 4.10**).

Finally, to validate this technique we determined whether the fixed and unfixed Th1 and Th2 samples had the gene expression profiles we would expect from the literature. We performed an unpaired T test comparing the fixed Th1 cells to the fixed Th2 cells, and compared the unfixed Th1 cells to the unfixed Th2 cells. The p-value cut-off was set at 0.05. We then looked at the top 20 most highly expressed genes in each cell type: fixed and unfixed Th1 cells, and fixed and unfixed Th2 cells (**Figure 4.11**). In the Th1 cells,

16 of the top 20 genes expressed in the fixed sample were also in the top 20 genes in the unfixed samples (**Figure 4.11 A**). Furthermore, *Ifng* was the most highly expressed gene in both the fixed and unfixed Th1 cells. Three of the genes that were found in the fixed Th1 top 20 were not present in the unfixed Th1 top 20 (*ligp1, Klrb1f, Twist1*). These genes were however present in the unfixed top 33. Three of the genes that were found in the unfixed Th1 top 20 were not present in the fixed Th1 top 20 (*Cldnd2, Cd86, Nkg7*). These genes were however present in the fixed top 31. Therefore, there was a large amount of overlap between the fixed and unfixed Th1 cells at the gene expression level, and many of the genes that were highly expressed by these cells are associated with Th1 gene expression profiles.

In the Th2 cells, 14 of the top 20 genes expressed in the fixed sample were also in the top 20 genes in the unfixed samples (**Figure 4.11 B**). *Il5, Il13* and *Il4* were all within the top 16 most highly expressed genes for both fixed and unfixed Th2 cells. Furthermore, the 3 most highly expressed genes were the same in both the fixed and unfixed Th2 cells (*Adamtsl3, Akr1c18, Il5*). Six genes that were found in the fixed Th2 top 20 were not present in the unfixed Th2 top 20 (*Rnf152, Nrgn, Il24, Mnp10, Mei4, Epas1*). These were however present in the unfixed top 35. Five of the genes that were found in the unfixed Th2 top 20 were not present in the fixed Th2 top 20 (*Dgkk, Gpr83, Prkcdbp, Hap1, Pdzrn3*), but these were present in the fixed top 52. Therefore, the fixed and unfixed Th2 cells were very similar at the gene expression level, and many of the genes that were highly expressed by these cells are associated with Th2 gene expression profiles.

4.4 Discussion

T helper cells secrete their hallmark cytokines, to regulate the proinflammatory responses of the immune system (Zhu et al., 2010), alongside IL-10 (Maynard and Weaver, 2008; Saraiva and O'Garra, 2010), which feeds back to reduce proinflammatory cytokine secretion and proliferation (Ouyang et al., 2011). However, each T helper cell subset does not form a homogeneous population of cells, in fact with regard to cytokine secretion the Th cell subsets are very heterogeneous. Therefore, to better understand the mechanisms involved in regulating *Il10* gene expression and hallmark cytokine gene expression, we wanted to set up a system to separate the Th subsets into subpopulations based on cytokine production and analyse the differential gene expression patterns within these populations using RNA-Seq.

We decided not to use reporter mice as a system for isolating different cytokine secreting Th cell subpopulation. This was because, to look at all the different Th cell subsets and their different cytokine secreting subpopulations, we would need to generate multiple dual reporter mice strains, which would need to be crossed to knockout mouse strains for future studies. These would require much time and money to generate, and this system could not be used on human samples. Furthermore, we discounted using single cell RNA-Seq for this study as, aside from the limitations of this newly developing technique, we want to be able to perform a comparison between protein production and mRNA expression. Single cell RNA-Seq only assesses cells at the mRNA level, and consequently this technique is not well suited for this project. Therefore, to separate Th cell subset subpopulations based on their protein profiles for analysis by RNA-Seq, we have designed and optimised a system for extracting viable mRNA for RNA-Seq analysis from intracellular cytokine stained samples.

4.4.1 Preparing samples for intracellular cytokine staining results in mRNA degradation

4.4.1.1 qPCR, but not RNA-Seq, can be performed on degraded samples

Using qPCR we found that the expression of hallmark cytokine genes in Th1 and Th2 cells was comparable between fixed (ICS prepared) samples and unfixed samples.

However, as suggested in the literature (Opitz et al., 2010), we found that mRNA from ICS samples was of low quality. This low quality did not have an affect on the result when analysing these samples by qPCR. Furthermore, other studies have found that this low quality does not seem to have an affect on the result when analysing these samples by microarray (Haller et al., 2006; Hodgin et al., 2010). However, this low quality dramatically affected the RNA-Seq results, as compared to high quality unfixed mRNA, and data from these fixed samples could not be analysed.

The ability to analyse low quality mRNA samples with qPCR or microarray may be a result of the way in which qPCR and microarray detect mRNA. qPCR and microarray work on a probe-based system. Probes for specific short sequences within a known molecule of mRNA are used to detect the presence of that mRNA molecule. Most of these probes are designed to detect sequences at the 3' end of mRNA molecules (Opitz et al., 2010). Therefore, even if an mRNA molecule is degraded, as long as the 3' end is intact then qPCR and microarray will be able to detect that molecule. As ribonucleases tend to degrade from the 5' end, it is possible to sidestep this issue by analysis of fixed samples with microarray. RNA-Seq however, works on a system that aligns all mRNA transcripts to the genome. If the mRNA molecules are degraded then material will be absent and cannot be aligned, the resulting under- and over- representation of genes will lead to unreliable and biased results (Sigurgeirsson et al., 2014). Therefore it is possible that qPCR and microarray can be performed on fixed samples because they work using a probe based system. However, if the mRNA is degraded then RNA-Seq cannot be performed on ICS samples.

4.4.1.2 Permeablisation of cells for intracellular cytokine staining leads to degradation of mRNA

To elucidate at which stage mRNA damage was occurring during the preparation of cells for ICS, we took mRNA samples for electropherogram analysis at each step. We found that, contrary to our expectation, it was the permeablisation stage rather than the fixation stage that resulted in the majority of damage to the mRNA samples. Although our samples did have slightly a lower RIN after fixation, and therefore extended periods of fixation may lead to mRNA damage. RNA-Seq analysis of the fixed (ICS prepared)

and unfixed samples revealed that the mRNA was being degraded, rather than fragmented, during the ICS protocol.

Therefore, permeablisation of the cell membranes during ICS resulted in the degradation of mRNA within the samples. Analysis of gene coverage revealed that the genes were being overrepresented at the 3' end and underrepresented at the 5' end. This suggested that the mRNA molecules were being degraded by 5' exonucleases upon permeablisation (Houseley and Tollervey, 2009). Where these RNases were coming from though is unknown; RNases are important in many physiological processes and are ubiquitous (Opitz et al., 2010). However, it is likely that the 5' exonucleases performing this degradation are of eukaryotic origins, as it is widely accepted that bacteria cannot degrade RNA in the 5' to 3' direction (Mathy et al., 2007). The permeablisation of the cells may allow RNases to access the cellular mRNA via two mechanisms. First, the permeablisation of the cellular membrane may allow enzymes from the extracellular environment to enter the cells and degrade the mRNA. Second, the permeablisation may lead to the lysis of intracellular compartments such as P bodies, mitochondria or the nucleus; all of which contain enzymes that are specialised in the degradation of RNA (Houseley and Tollervey, 2009). Either way, upon permeablisation of the cells, mRNA is rapidly and extremely degraded in a 3' biased manner, resulting in samples that were not viable for RNA-Seq analysis.

4.4.2 The addition of RNase inhibitors prevents mRNA degradation in intracellular cytokine stained samples

As RNases appeared to be causing mRNA degradation within our ICS samples, we aimed to find a way to prevent the activity of these enzymes. Ribonuclease inhibitor (RI) is a mammalian 50KDa protein that forms extremely strong protein-protein interactions with certain RNases and renders these enzymes inactive (Dickson et al., 2005). RI binds to RNases non-covalently at a 1:1 ratio. Therefore, RI can be used to prevent the degradation of RNA by RNases. Furthermore, a series of recent publications that have successfully prepared human intracellularly stained samples for microarray or RNA-Seq, used RNase inhibitors (Hrvatin et al., 2014a; Hrvatin et al., 2014b; Kenty and Melton, 2015). To test if RNases were the cause of the mRNA degradation we observed in our ICS samples, and to see if we could block the activity of these enzymes,

we added RNase inhibitors throughout the ICS protocol. We found that the use of RNase inhibitors resulted in high RINs when assessed by electropherogram, and when analysed by RNA-Seq, the gene coverage in the fixed samples was equivalent to that seen in unfixed samples. Therefore, we can prevent RNA degradation during ICS by adding RNase inhibitors.

4.4.3 Fixed and unfixed Th1 and Th2 cells are equivalent and have characteristic gene expression profiles

Having established a protocol for extracting viable mRNA from fixed (ICS) samples, we wanted to ensure that the mRNA profiles from these samples were comparable to samples that had not undergone ICS. Therefore we performed RNA-Seq on fixed and unfixed Th1 and Th2 cells to assess the similarities and differences between these samples.

4.4.3.1 There is no significant difference between fixed and unfixed Th1 cells at the global gene expression level

A correlation between all the genes expressed in fixed Th1 cells and unfixed Th1 cells showed that there was almost no difference between the samples. This highly positive correlation was reproducibly seen across three biological replicate experiments. This is in keeping with Hrvatin et al., who showed gene expression between fixed and unfixed samples was very similar when preparing cells in the presence of RNase inhibitors (Hrvatin et al., 2014a). We consistently got high R^2 values when comparing fixed and unfixed Th1 cells. To further elucidate any differences between the three repeats, we looked at genes that were only present in the fixed samples (6%), or only present in the unfixed samples (1%). We then assessed whether any genes were consistently 'lost' or 'gained' upon fixation of the samples. We found little consistency in the repeats with regard to the loss or gain of genes, and that mostly these genes appeared to be random. This suggested, as one would expect (Marioni et al., 2008), that there were insignificant random differences between each of the samples prepared for RNA-Seq, but that this was not affected by fixation of the samples. This was supported by an unpaired T test

that revealed there was no significant difference between the fixed and unfixed Th1 cells.

4.4.3.2 There is very little difference between fixed and unfixed Th2 cells at the global gene expression level

A correlation between all the genes expressed in fixed Th2 cells and unfixed Th2 cells showed that there was very little difference between the samples. As with the Th1 cells, this highly positive correlation was reproducibly seen across three biological replicate experiments, and consistently high R^2 values were found when comparing fixed and unfixed Th2 cells. To further elucidate any differences between the three repeats, we assessed genes that were only present in the fixed samples (2%), or only present in the unfixed samples (6%). When comparing these genes to see if any genes were consistently 'lost' or 'gained' upon fixation of the samples, we found that there were only a few genes consistently gained or lost, and that mostly these genes seemed to be random. This was supported by an unpaired T test that revealed that only 0.17% of the total genes expressed in the samples was significantly different between the fixed and unfixed Th2 cells. Furthermore, when looking at genes that had a greater than two fold-change difference, the number dropped to below 0.1%. Therefore there was obviously a minor difference between the fixed and unfixed Th2 cells, however at the expression level this difference was only marginally significant and no genes seemed to be consistently lost or gained.

4.4.3.3 Fixed and unfixed Th1 and Th2 cells are comparable in expression for highly represented genes

After establishing that there was little difference between the fixed and unfixed Th1 and Th2 cells at the whole gene expression level we wanted to ascertain that the samples were comparable at the single gene level. Furthermore we wanted to ensure that our *in vitro* cultured Th cells were expressing the genes one would expect from the literature. Therefore we decided to look at the expression level of seven key cytokine and transcription factor genes that are associated with Th1 or Th2 cells (Zhu et al., 2010).

To define Th1 cells we chose to look at the expression of *Ifng* and *Tbx21*, and to define Th2 cells we chose *Il4, Il5, Il13* and *Gata3,* alongside *Il10*. Looking at the expression of these genes, we firstly noted that the there was very little difference in normalised expression levels between the three repeats (as shown by the error bars). Secondly, we saw that the expression levels between the fixed and unfixed samples was almost identical, and finally we observed the gene expression profiles we would expect. Both fixed and unfixed Th1 cells expressed high levels of the hallmark cytokine *Ifng* and the master transcription factor *Tbx21*, while both fixed and unfixed Th2 cells expressed high levels of the hallmark cytokines *Il4, Il5* and *Il13* and the hallmark transcription factor *Gata3*. *Gata3* was more highly expressed in the Th2 cells than the Th1 cells, but this was not as noticeably different as it was for *Tbx21*. The relatively high levels of *Gata3* expression in Th1 cells may be due to the important role GATA3 plays in CD4+ T cell development (Wei et al., 2011) and as a pioneer transcription factor (Wei et al., 2011; Zhang et al., 2012). *Il10* was expressed to almost equal amounts in both the Th1 and Th2 cells, matching what we saw at the protein level. Therefore we concluded that the fixed and unfixed Th1 and Th2 cells were repeatable and comparable, and they expressed the genes we would expect from the literature.

4.4.3.4 Fixed and unfixed Th1 and Th2 cells express subset specific genes

Finally, we took an unbiased approach to look at the differences between the fixed and unfixed samples at the single gene level. Performing an unpaired T test between the fixed Th1 and Th2 cells and the unfixed Th1 and Th2 cells, we looked at the top 20 most highly expressed genes in each cell type. We found a large amount of overlap in these genes between the fixed and unfixed Th1 cells and also between the fixed and unfixed Th2 cells. Furthermore, we found that many of the most highly expressed genes have been reported to be expressed in the corresponding cell type.

In both the fixed and unfixed Th1 samples, *Ifng*, which is the Th1 hallmark cytokine, was the most highly expressed gene. In the Th1 samples several members of the CD94/Natural Killer cell surface protein family (Killer cell lectin-like receptors) were present in both the fixed and unfixed top 20, namely *Klrc1, Klrk1* and *Klrd1*. *Klrb1f*, which was in the fixed Th1 sample top 20, is the 25th most highly expressed in the unfixed Th1 sample. Members of this family of molecules are specifically expressed on

the surface of Th1 cells (Graham et al., 2007), and it has been suggested that they are involved in the costimulation of these cells (Meyers et al., 2002). Two members of the membrane spanning 4A family (*Ms4a4b* and *Ms4a4c*) were found in both Th1 samples. Chandra/MS4a4B is reportedly expressed on Th1 cells but not Th2 cells (Venkataraman et al., 2000), and overexpression of MS4a4B results in TCR-induced Th1 cytokine expression in primary CD4+ T cells (Xu et al., 2006). MS4a4C is closely related to 4B, and though it has more widely distributed cellular expression, it has a similar expression pattern to 4B in T cells (Xu et al., 2006). Furthermore, the interferon-inducible gene *Ifi204* was present in both Th1 samples, which has been associated with differentiated Th1 cells. This gene is found in resting naive CD4+ T cells, its expression is downregulated as cells are TCR stimulated and driven in Th1 and Th2 conditions, however high expression returns as the Th1 cells but not Th2 cells differentiate (Lu et al., 2004). Other Th1 related factors found in the fixed Th1 top 20 included *Ligp1* and *Twist1*. *Ligp1* encodes an IFN-stimulated GTPase that is part of the p47 family, the expression of which is dramatically upregulated by IFNγ (Stockinger, 2000-). NFAT and IL-12 signalling via STAT4 has been shown to upregulate *Twist1* expression in Th1 cells (Niesner et al., 2008; Pham et al., 2012), and the levels of this factor are increased upon repeated TCR stimulation (Niesner et al., 2008). Therefore this factor is thought to be associated with chronic Th1 inflammation (Haftmann et al., 2015), and acts as a negative feedback to T-bet binding at the *Ifng* locus (Pham et al., 2012) resulting in reduced IFNγ production (Niesner et al., 2008; Pham et al., 2012).

In both the fixed and unfixed Th2 cells, all three of the hallmark cytokine genes, *Il4, Il5* and *Il13*, were highly expressed. Furthermore, *Il24*, which is a member of the IL-20 subfamily of the IL-10 family of cytokines (Ouyang et al., 2011), and is located upstream of *Il10* on chromosome 1, was also highly expressed in these cells. IL-24, also known as FISP or IL-4-induced secreted protein, is selectively expressed in differentiated Th2 cells, and its expression requires both TCR signalling and STAT6-dependent IL-4 signalling (Schaefer et al., 2001). Another IL-4 and IL-13 regulated gene that is expressed in both the Th2 samples was *Hs3st1*. This sulphotransferase has been shown to be upregulated in epithelium-derived cells by IL-4/IL-13 (Takeda et al., 2010), though its expression in Th2 cells themselves has not been reported. However, as Th2 cells respond to IL-4 it is possible that this gene is upregulated upon Th2 cell differentiation. *Cxcl3* was highly expressed in both Th2 samples, which is in keeping with findings that this chemokine is positively regulated by GATA3 (Sasaki et al.,

2013). Finally, *Eaps1*, which was the 20th most highly expressed gene in the fixed Th2 cells, and 25th most highly expressed gene in the unfixed Th2 cells, has also been associated with Th2 cells in the literature. GATA3 binds the *Epas1* gene (Horiuchi et al., 2011), and it is also a direct target of STAT6 (O'Shea et al., 2011). Furthermore, as Th2 cells differentiate, the levels of Epas1 have been shown to increase (Lu et al., 2004).

4.4.4 Conclusions and future plans for separating T helper cells into different cytokine expressing populations for analysis by RNA-Seq

We have established a method for separating Th cell subset subpopulations based on their intracellular cytokine protein profiles for analysis by RNA-Seq. We have designed a protocol in which viable mRNA for RNA-Seq analysis can be extracted from ICS samples when prepared in the presence of RNase inhibitors. RNA-Seq analysis reveals that the gene expression profiles of fixed ICS Th1 and Th2 samples were as expected from the literature. For future experiments we want to separate different T helper cell subpopulations based on intracellular cytokine staining, and then analyse these samples using RNA-Seq, using the protocol shown in **Figure 4.4**.

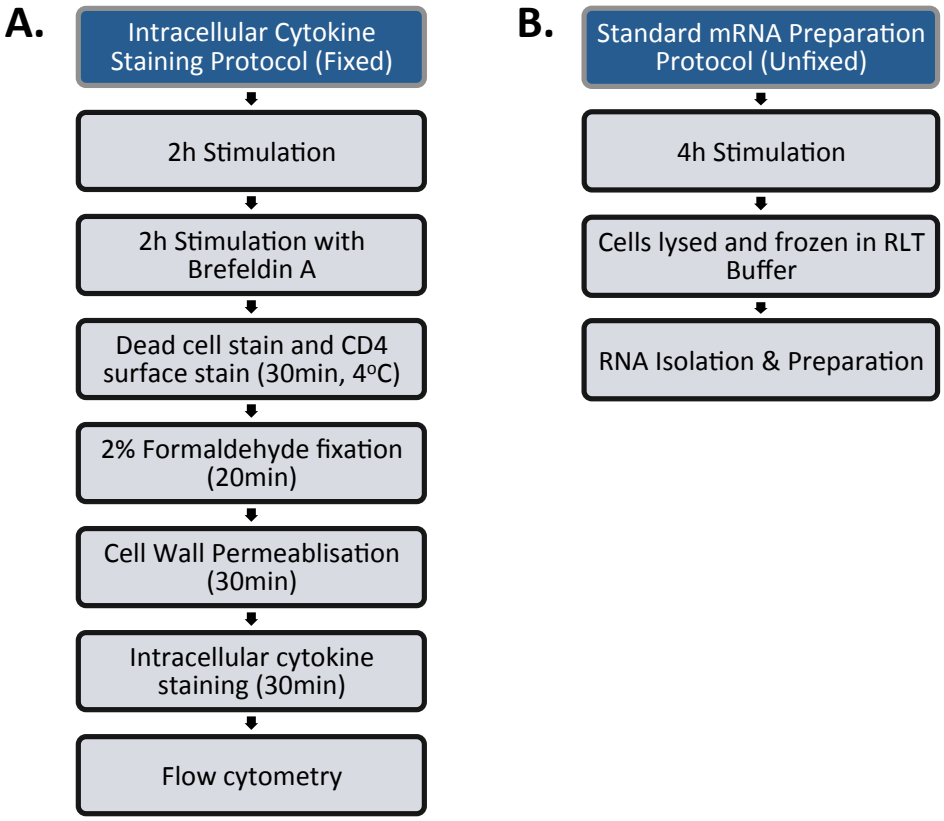


Figure 4.1 The standard protocols for intracellular cytokine staining and for mRNA extraction

A. Polarised T helper cells were restimulated for 4 hours (with BfA for the second 2 hours). They were then stained for dead cells and surface markers, fixed, permeablised and intracellular cytokine stained. Samples were then separated into different cytokine producing populations using FACS. **B.** Polarised T helper cells were restimulated for 4 hours. They were then washed and transferred to RLT buffer, which lysed the cells, and frozen. RNA was later extracted using the Qiagen RNeasy kit.

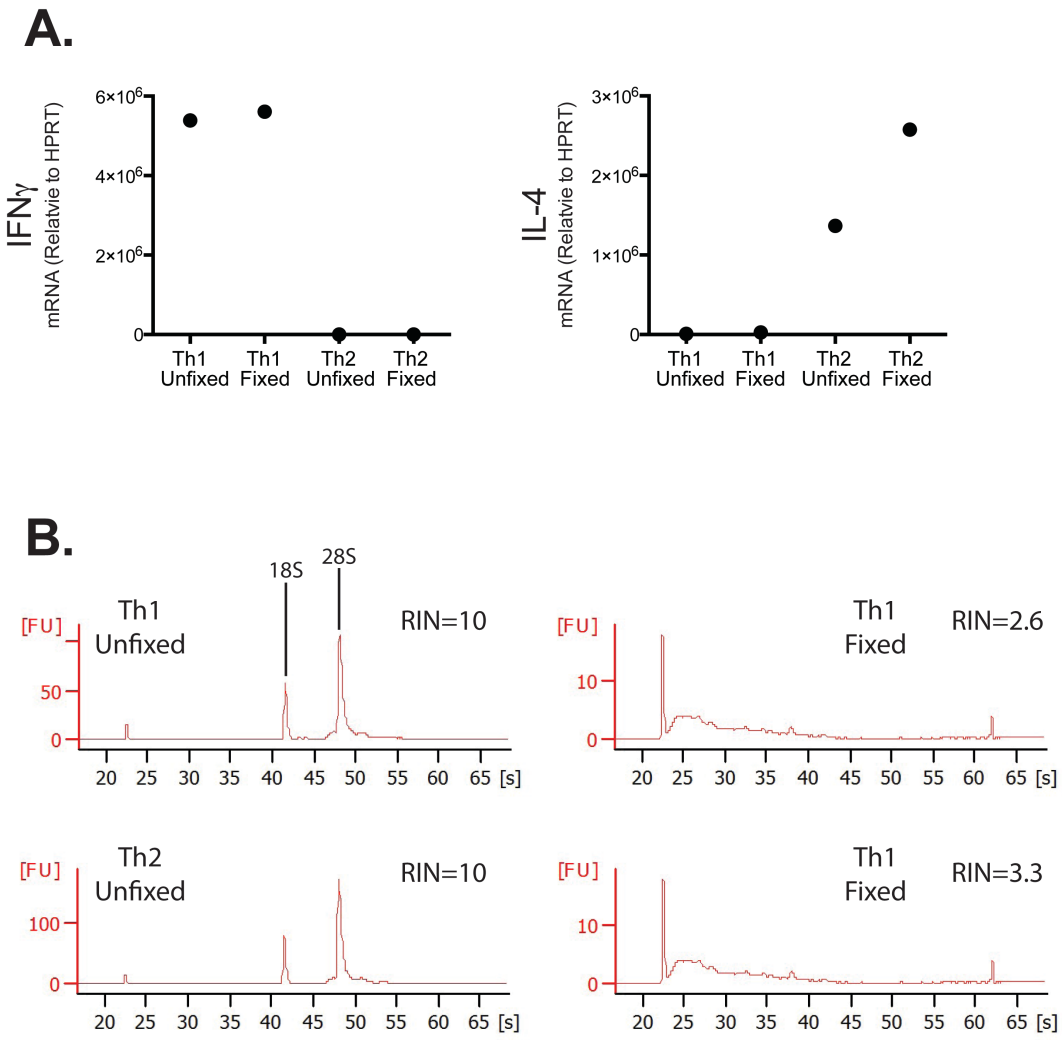


Figure 4.2 mRNA extracted from Th1 and Th2 cells before and after fixation and permeablisation for intracellular cytokine staining

C57BL/6 naive CD4+CD62L+CD44loCD25- cells were driven with plate-bound anti-CD3 and soluble anti-CD28, and polarised to Th2 cells with IL-4, IL-2, anti-IL-12 and anti-IFNγ, Th1 cells with IL-12, anti-IL-4 and IL-27. After 7 days of polarisation *in vitro* cells were harvested and restimulated for 4 hours, and either lysed and stored in RLT buffer for RNA extraction with Qiagen RNeasy Kit or fixed with formaldehyde, washed and stored for RNA extraction with Qiagen FFPE RNA extraction Kit. **A.** Graphs of cytokine mRNA measured by qPCR relative to HPRT. **B.** Isolated RNA quality assessed using 2100 Biolanalyser (Agilent Technologies, Inc.). Simulated electropherogram suggests minimal degradation of total RNA based on 10S and 28S ribosomal RNA bands. Representative of one experiment.

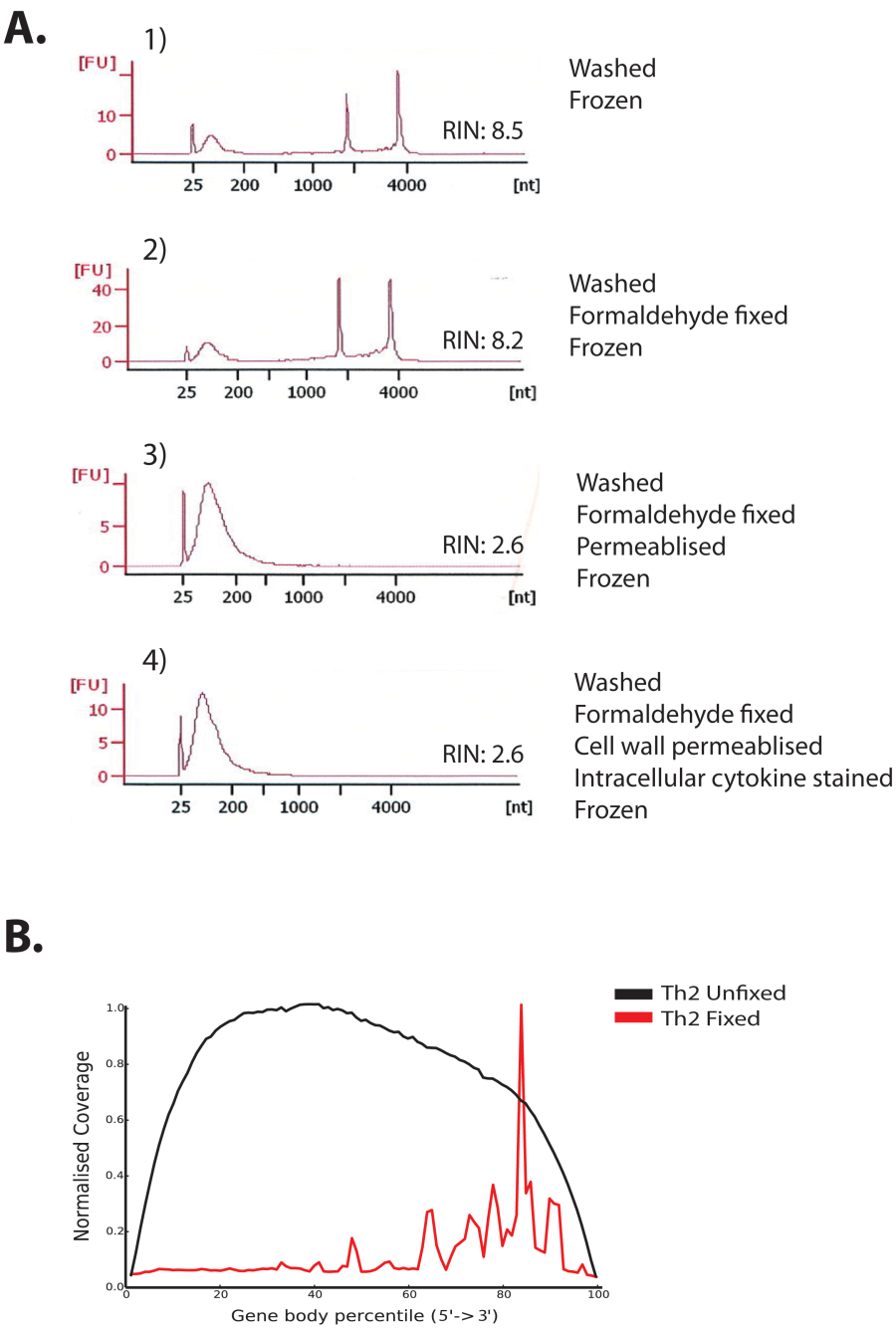


Figure 4.3 Permeablisation degrades RNA in samples prepared using the standard intracellular cytokine staining protocol

C57BL/6 naive CD4+CD62L+CD44loCD25- cells were driven with plate-bound anti-CD3 and soluble anti-CD28, and polarised to Th2 cells with IL-4, IL-2, anti-IL-12 and anti-IFNγ. After 7 days of polarization *in vitro* cells were restimulated as described in Materials and Methods. **A.** Cells were taken at different times throughout the ICS protocol and stored for RNA extraction with the Qiagen FFPE RNA extraction kit. Isolated RNA quality assessed using 2100 Biolanalyser (Agilent Technologies, Inc.). **B.** RNA samples from the first group (Washed, Frozen) and from the last group (ICS Fixed) were single-end sequenced using TruSeq chemistry on a HiSeq 2500 (Illumina). Graph shows relative RNA-Seq coverage of all annotated transcripts. Representative of one experiment.

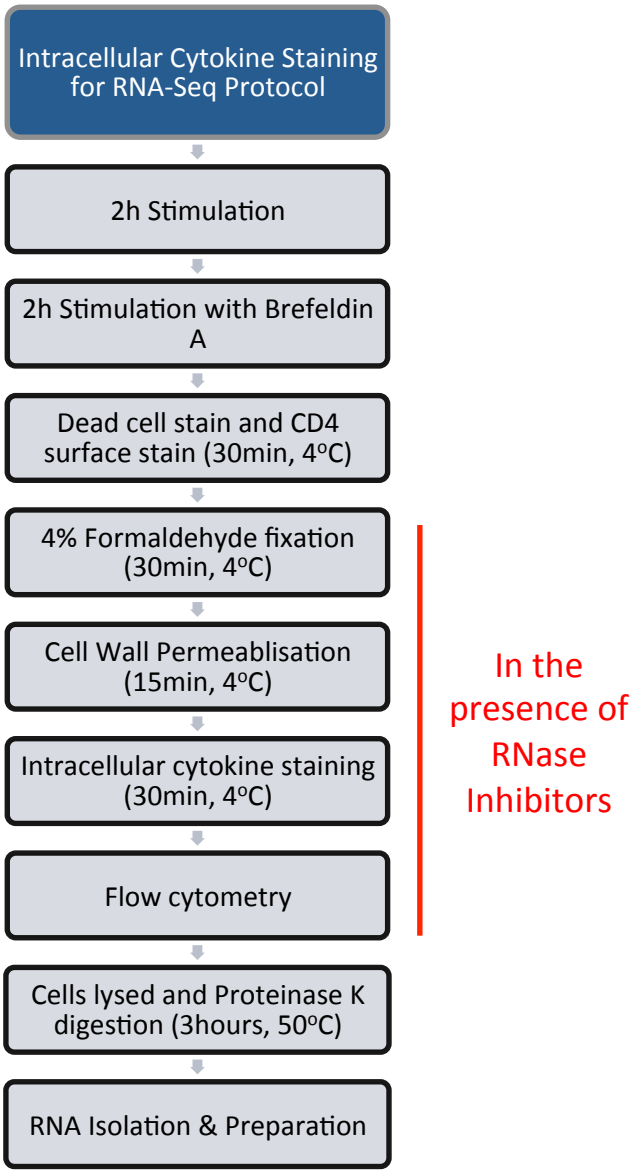


Figure 4.4 The addition of RNase inhibitors during the intracellular cytokine staining protocol to ensure high quality mRNA for RNA-Seq

Polarised Th cells were restimulated for 4 hours (with BfA for the second 2 hours). Cells were then stained for dead cells and surface markers. Fixation, permeablisation and intracellular cytokine staining were performed in the presence of RNase inhibitors. Samples were then separated into different cytokine producing populations using FACS in the presence of RNase inhibitors. The samples were then lysed and proteinase K digested before RNA is extracted using the Qiagen FFPE RNA extraction kit.

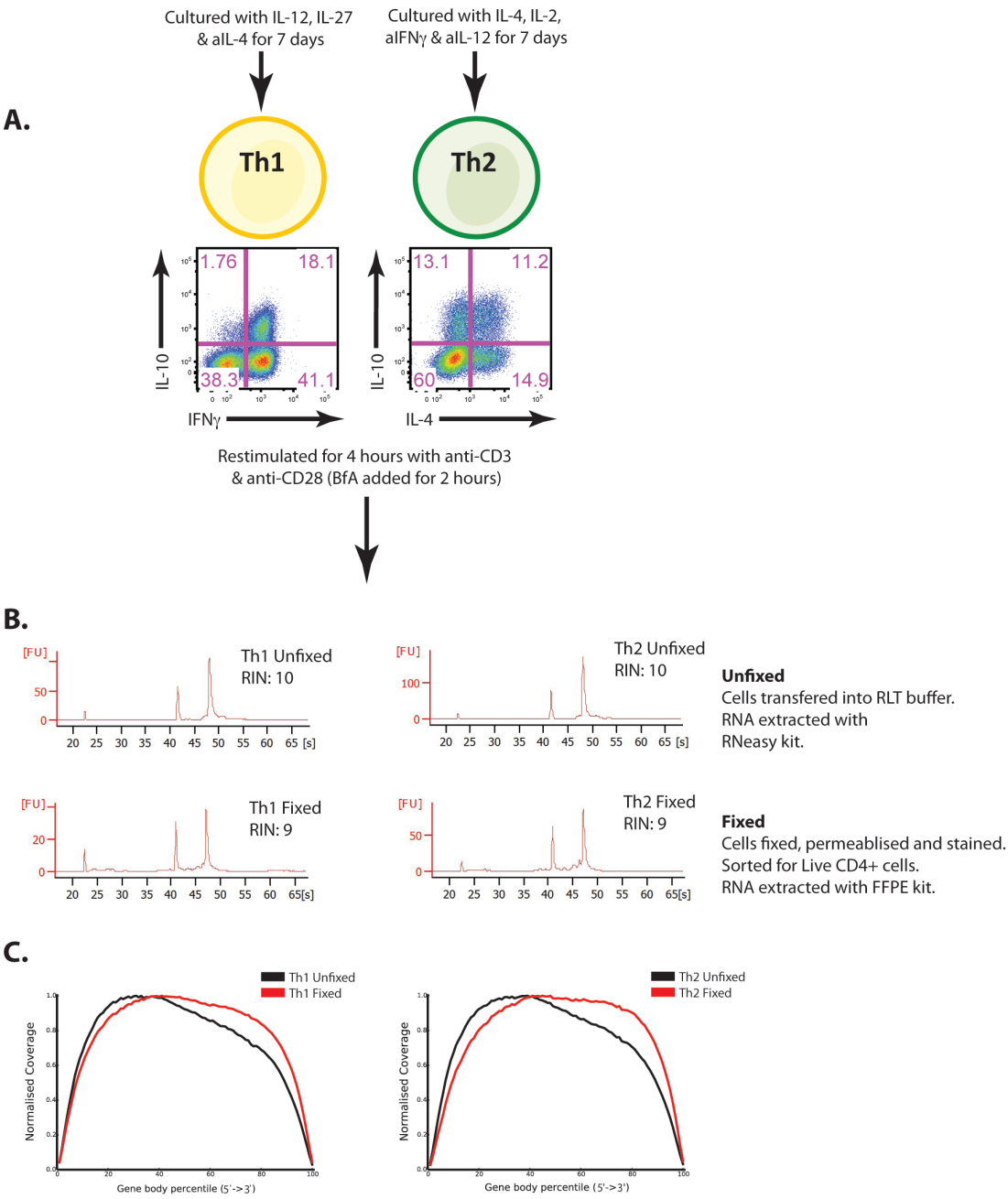


Figure 4.5 The addition of RNase inhibitors prevents mRNA degradation caused by permeablisation when staining for intracellular cytokines

C57BL/6 naive CD4+CD62L+CD44loCD25- cells were driven with plate-bound anti-CD3 and soluble anti-CD28, and polarised to Th2 cells with IL-4, IL-2, anti-IL-12 and anti-IFNγ or Th1 cells with IL-12, IL-27 and anti-IL-4. Cells were assessed after 7 days of polarization *in vitro*. **A.** Plots of flow cytometric analysis of intracellular cytokine staining, cells restimulated as described in Materials and Methods. Numbers show percentage of live CD4+ cells. **B.** mRNA isolated and analysed by electropherogram from Th1 and Th2 cells using standard protocol (Unfixed) or following fixation, staining and sorting (Fixed). **C.** Samples were prepared and single-end sequenced using TruSeq chemistry on a HiSeq 2500 (Illumina). Relative RNA-Seq coverage of all annotated transcripts. Representative of three experiments.

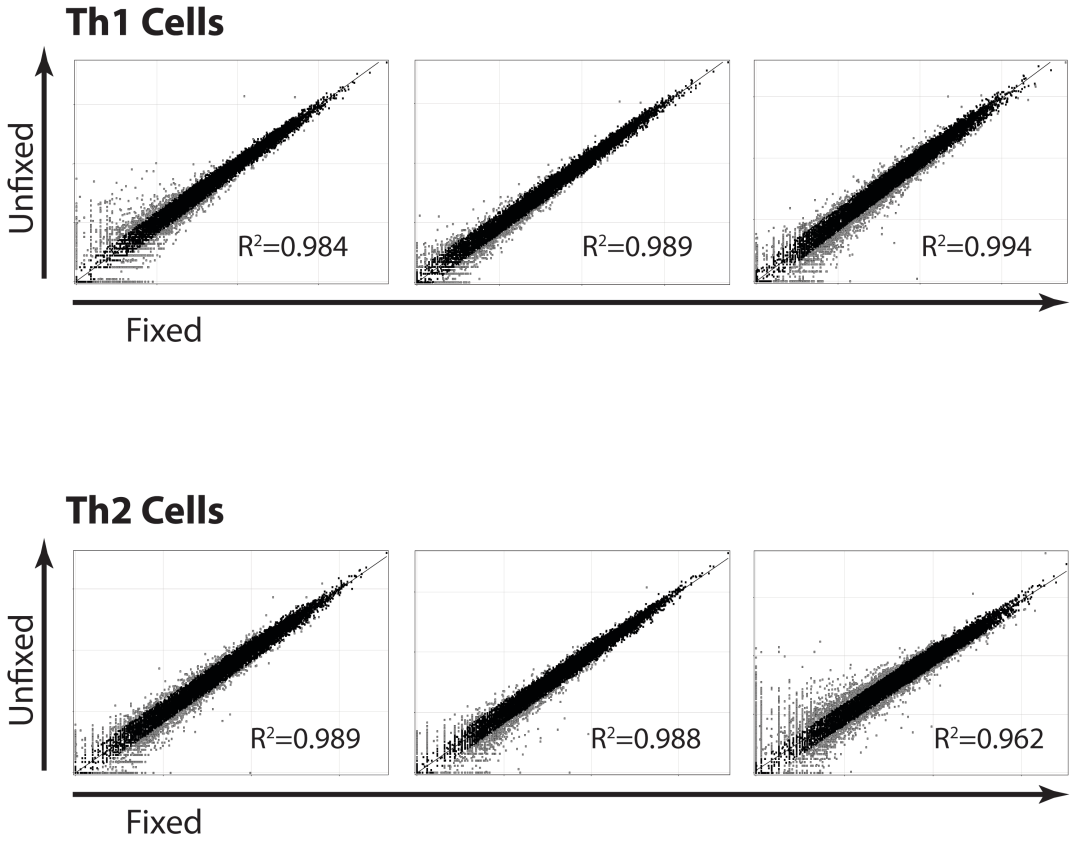


Figure 4.6 Quantitative comparison between fixed and unfixed Th1 and Th2 samples

Using Strand NGS software reads were aligned to the transcriptome & Genome (mm10, RefSeq annotation, 95% identity, max 5% gaps, 1 read only if duplicate) and normalisation with DeSeq and no Baseline. The correlation coefficients of the per-gene expression values in fixed and unfixed samples were reported for three biological replicate experiments in the respective graphs. The high correlations demonstrated the similarity between the fixed and unfixed samples. Grey dots represent genes that are 2-fold differentially expressed between the fixed and unfixed samples.

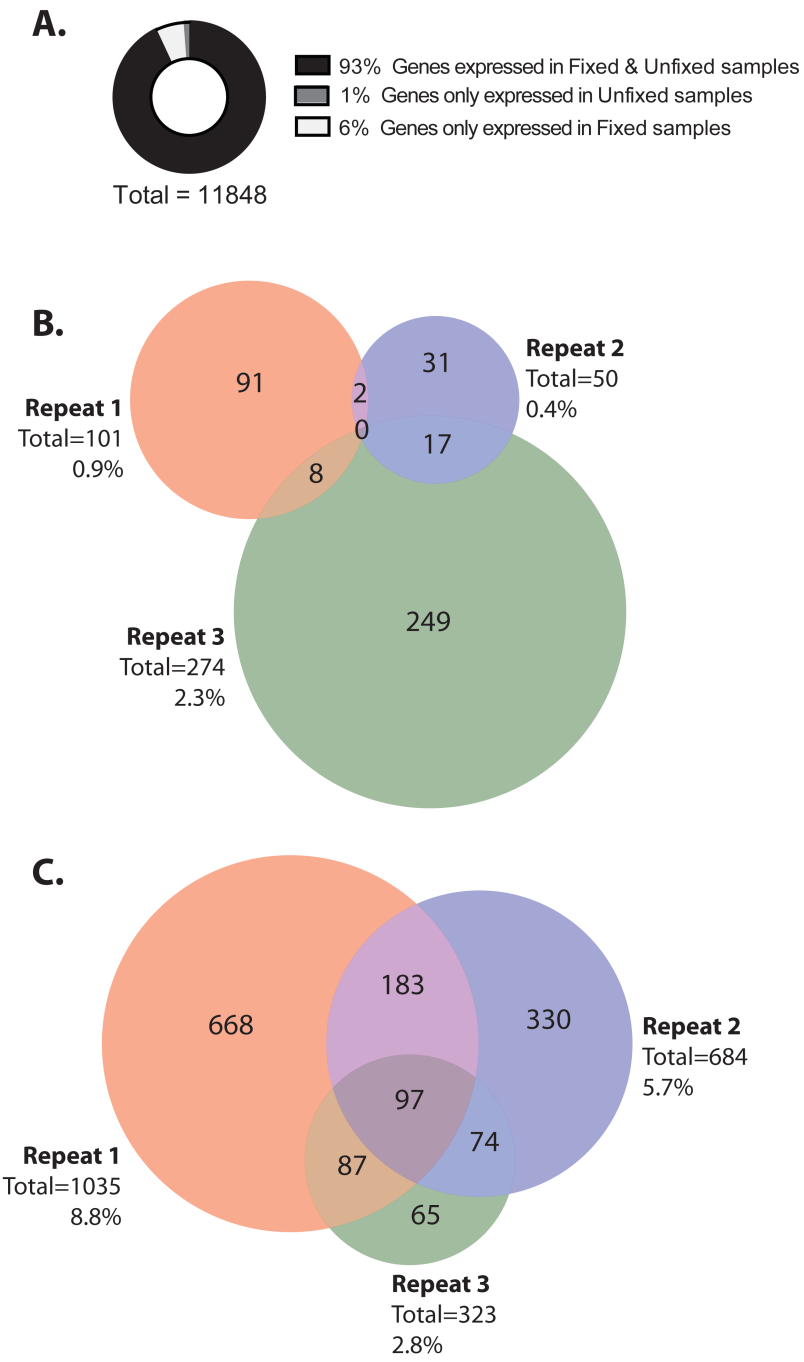


Figure 4.7 Differential gene expression of fixed and unfixed Th1 samples

Analysed using Strand NGS software as described in Materials and Methods. Upper cut off 983409, lower cut off 10 12,652 genes). **A.** The doughnut plot represents an average of the total number of genes expressed over the three Th1 repeat experiments. Black – the number of genes that were expressed in both the fixed and unfixed samples. White - the proportion of genes expressed in the fixed samples that were not present in the unfixed samples. Grey - the proportion of genes expressed in the unfixed samples that were not present in the fixed samples. **B & C.** For each repeat a list of genes that were present in the unfixed samples but not in the fixed samples (**B**) or in the fixed samples but not in the unfixed samples (**C**) were created. These three lists were then overlapped for form a Venn. The number in the centre of the Venn represents the number of genes that were always present in the unfixed samples and not in the fixed samples (**B**) or always present in the fixed samples and not in the unfixed samples (**C**). The percentages represent the portion this number of genes is within the total number of genes expressed in that sample.

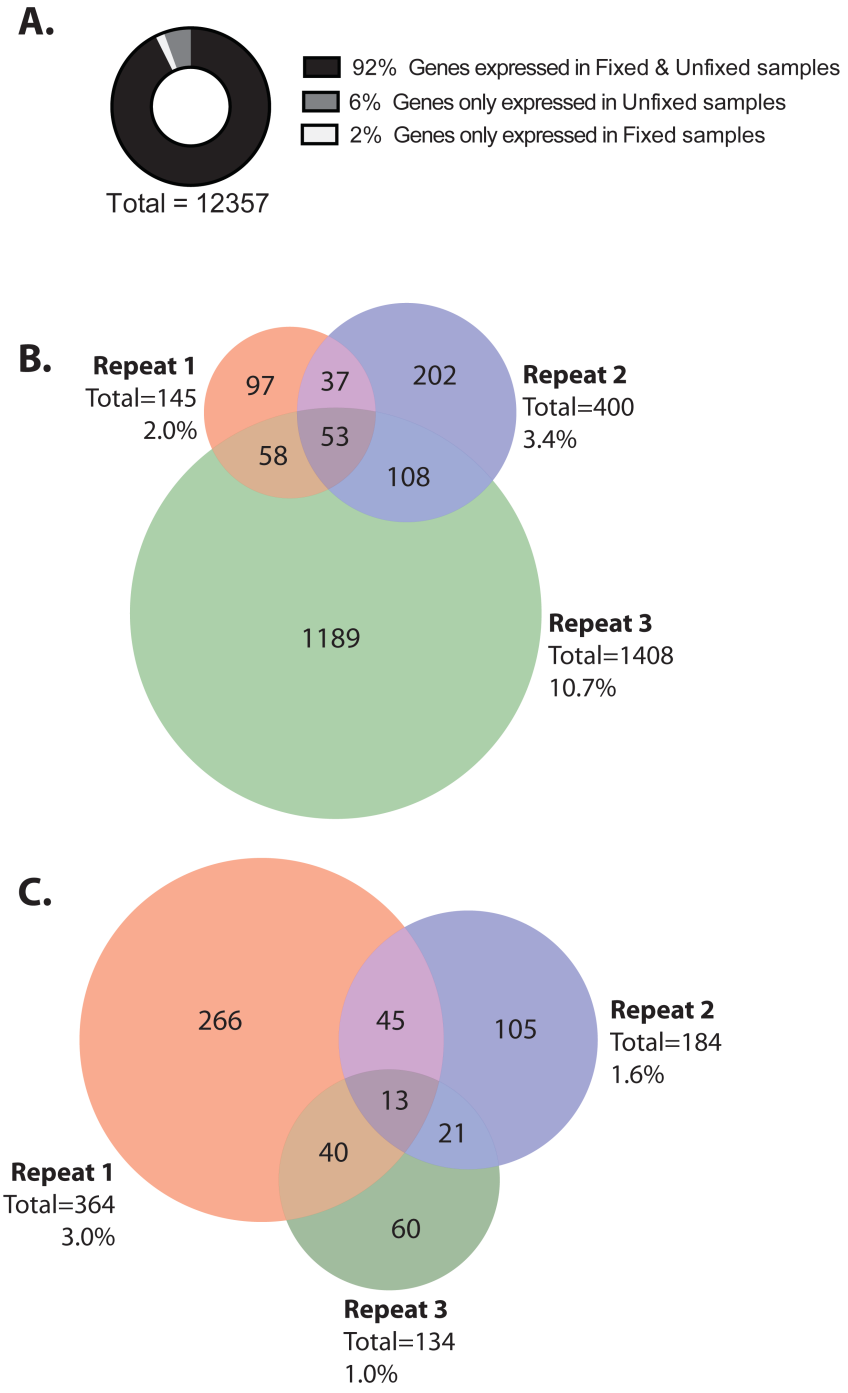
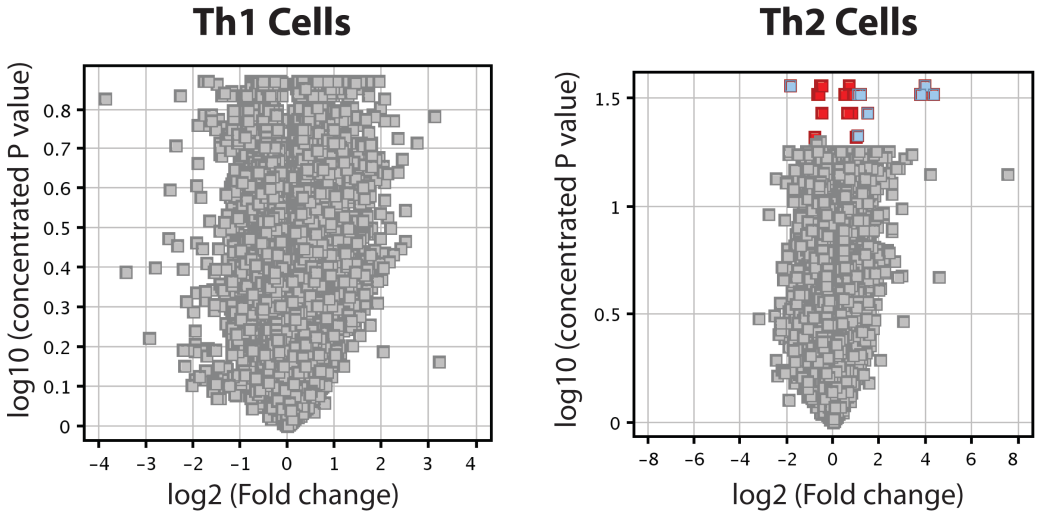


Figure 4.8 Differential gene expression of fixed and unfixed Th2 samples

Analysed using Strand NGS software as described in Materials and Methods. Upper cut off 983409, lower cut off 10 (12,652 genes). **A.** The doughnut plot represents an average of the total number of genes expressed over the three Th2 repeat experiments. Black – the number of genes that were expressed in both the fixed and unfixed samples. White - the proportion of genes expressed in the fixed samples that were not present in the unfixed samples. Grey - the proportion of genes expressed in the unfixed samples that were not present in the fixed samples. **B & C.** For each repeat a list of genes that were present in the unfixed samples but not in the fixed samples (**B**) or in the fixed samples but not in the unfixed samples (**C**) were created. These three lists were then overlapped for form a Venn. The number in the centre of the Venn represents the number of genes that were always present in the unfixed samples and not in the fixed samples (**B**) or always present in the fixed samples and not in the unfixed samples (**C**). The percentages represent the portion this number of genes is within the total number of genes expressed in that sample.

A.



B.

	P all	P < 0.05
FC all	12652	0
FC > 1.1	8751	0
FC > 2.0	634	0
FC > 3.0	142	0

	P all	P < 0.05
FC all	12652	21
FC > 1.1	9168	21
FC > 2.0	724	8
FC > 3.0	160	4

Figure 4.9 Differential gene expression between fixed and unfixed Th1 and Th2 samples

Analysed using Strand NGS software as described in Materials and Methods. Upper cut off 983409, lower cut off 10 (12,652 genes). **A.** The volcano plot shows the magnitude of differential expression between fixed and unfixed Th1 cells, or fixed and unfixed Th2 cells. Each dot represents gene transcripts that had detectable expression in both tissues. Genes that were differentially expressed between fixed and unfixed samples at $P < 0.05$ were indicated by coloring. Blue indicates > 2.0 fold change in expression, red indicated < 2.0 fold change in expression. **B.** The tables show the actual number of genes differently expressed, as illustrated in the volcano plots. Unpaired T test, asymptomatic P-value computation, Benjamini Hochberg multiple testing correction.

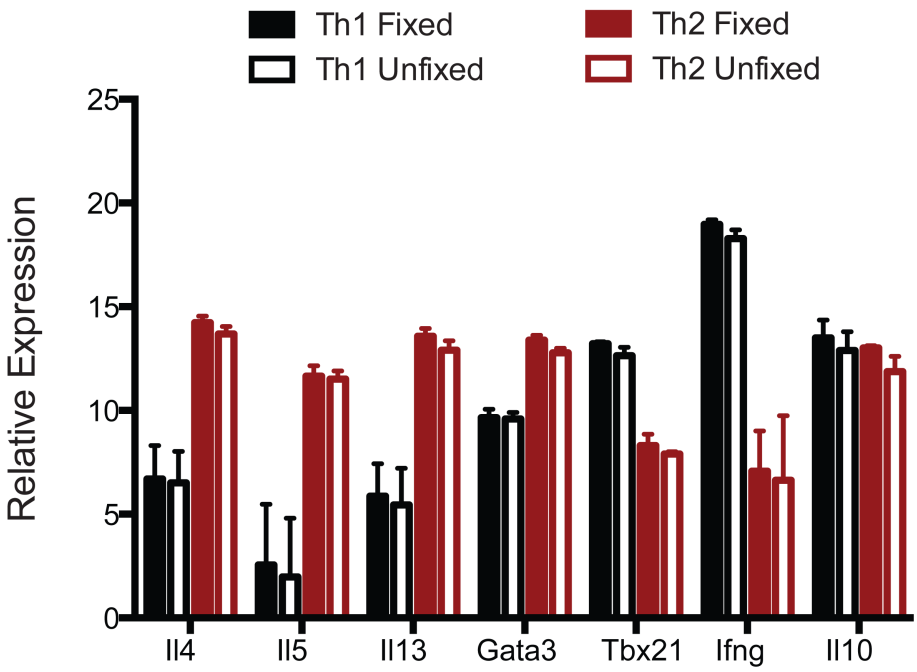


Figure 4.10 Hallmark cytokine and IL-10 gene expression in Th1 and Th2 cells, between fixed and unfixed samples

Analysed using Strand NGS software as described in Materials and Methods. Upper cut off 983409, lower cut off 10 (12,652 genes). Relative expression was averaged across the three biological repeats, with ± SD representing the difference between experiments.

A. Th1 Cells

Fixed

Log FC	Gene
11.89	*Ifng*
9.11	*Gm14446*
8.76	*Iigp1*
8.73	*Klrc1*
8.71	*Xcl1*
7.76	*Klrk1*
7.72	*Serpinb5*
7.54	*Klrd1*
7.22	*Ms4a4b*
7.09	*Ifi204*
7.07	*Kit*
7.03	*Plac8*
7.01	*Ccl9*
7.00	*BC094916*
6.99	*Klrb1f*
6.92	*Twist1*
6.90	*Pydc3*
6.80	*Ly6c2*
6.70	*Pydc4*
6.62	*Ms4a4c*

Unfixed

Log FC	Gene
11.65	*Ifng*
8.98	*Klrc1*
8.58	*Klrk1*
8.29	*Xcl1*
7.97	*Serpinb5*
7.69	*Klrd1*
7.15	*Gm14446*
6.83	*Pydc3*
6.83	*Ms4a4b*
6.77	*Ccl9*
6.76	*Ms4a4c*
6.49	*Ly6c2*
6.36	*Ifi204*
6.35	*Cldnd2*
6.31	*Cd86*
6.19	*Nkg7*
6.16	*BC094916*
6.13	*Plac8*
5.85	*Pydc4*
5.76	*Filip1*

B. Th2 Cells

Fixed

Log FC	Gene
9.74	*Adamtsl3*
9.63	*Akr1c18*
9.11	*Il5*
9.10	*Hs3st1*
7.89	*Grp*
7.87	*Cacna1c*
7.83	*Rnd3*
7.77	*Rnf152*
7.76	*Sln*
7.71	*Il13*
7.68	*Nrgn*
7.54	*Il4*
7.51	*Cxcl3*
7.26	*Il24*
6.99	*Nts*
6.99	*Mmp10*
6.90	*Cyp11a1*
6.88	*4930506M07Rik*
6.69	*Mei4*
6.67	*Epas1*

Unfixed

Log FC	Gene
9.53	*Il5*
9.12	*Akr1c18*
9.06	*Adamtsl3*
7.97	*Grp*
7.85	*Dgkk*
7.75	*Hs3st1*
7.67	*Sln*
7.46	*Il13*
7.44	*4930506M07Rik*
7.30	*Nts*
7.23	*Gpr83*
7.22	*Rnd3*
7.22	*Cxcl3*
7.19	*Cacna1c*
7.19	*Cyp11a1*
7.18	*Il4*
7.08	*Prkcdbp*
7.08	*Cuzd1*
6.94	*Hap1*
6.93	*Pdzrn3*

Common genes

Figure 4.11 The top 20 genes most highly expressed in fixed and unfixed Th1 and Th2 cells

Analysed using Strand NGS software as described in Materials and Methods. Upper cut off 983409, lower cut off 10 (12,652 genes). Log fold change of gene expression was calculated by comparing the fixed or unfixed Th1 cells to the Th2 cells. The top 20 genes that were most differentially expressed in one cell type over the other are displayed here. Grey boxes highlights genes that overlap in the top 20 for that cell type between fixed and unfixed.

Chapter 5. RNA-Seq analysis of differential gene expression in Th1 cells driven in the presence or absence of IL-27, and, in depth analysis of the different intracellular cytokine producing subpopulations within these Th1 and Th1 + IL-27 subsets

Chapter 5 (A) RNA-Seq analysis of differential gene expression in bulk populations of Th1 cells driven in the presence or absence of IL-27

5.1 (A) Background

Cytokines play a central role in determining and driving Th cell differentiation, and in controlling IL-10 production (O'Garra, 1998). Downstream of multiple cytokine receptors STAT and SMAD proteins have been shown to affect IL-10 expression (Batten et al., 2008; Blokzijl et al., 2002; Fitzgerald et al., 2007; Kitani et al., 2003; Saraiva et al., 2009; Stumhofer et al., 2007). *In vitro* differentiated Th2 cells make IL-10 (Fiorentino et al., 1989) and the factors that drive Th2 differentiation, namely IL-2 and IL-4, are required for IL-10 expression. Th1 cells, however, require alternative or additional cytokine signals alongside their differentiating cytokine IL-12 to initiate maximal IL-10 expression. We describe here mechanisms to induce reliable and robust IL-10 production by Th1 cells *in vitro*.

IL-12, which is predominantly produced by macrophages and DCs, is the main factor in driving Th1 cell differentiation and IFNγ production (Hsieh et al., 1993b). However, IL-27, the dominant source of which is myeloid populations (Yoshida and Hunter, 2015), can also activate STAT1 and T-bet to stimulate responsiveness to IL-12 and promote IFNγ production (Chen et al., 2000a; Lucas et al., 2003; Takeda et al., 2003). However, IL-12 and IL-27 have differential effects on Th1 cells. IL-27 not only promotes Th1 immunity, but also has a role in limiting overactive immune responses (reviewed in (Yoshida and Hunter, 2015)). IL-27 receptor KO mice develop lethal immune pathology associated with elevated IFNγ from CD4+ T cells (Villarino et al., 2003). It is now understood that IL-27 suppresses pathogenic Th1 responses by promoting CD4+ T cell IL-10 production (Anderson et al., 2009; Awasthi et al., 2007; Batten et al., 2008; Fitzgerald et al., 2007; Freitas do Rosario et al., 2012; Stumhofer et al., 2007; Torrado et al., 2015). The mechanisms by which IL-27 drives IL-10 production by CD4+ T cells are complex and not fully understood. Alongside STAT1 and STAT3 signalling (Stumhofer et al., 2007), costimulation of ICOS has been proposed to be involved in these events (Pot et al., 2009). Furthermore, IL-27 has been shown to induce the

expression of c-Maf and IL-21 (Pot et al., 2009), both of which have been suggested to induce IL-10 (Spolski et al., 2009; Xu et al., 2009). Additionally, IL-27 has been shown to induce interaction of c-Maf with AhR to promote IL-10 production from type 1 regulatory (Tr1) cells (Apetoh et al., 2010). However, the mechanisms that transcriptionally regulate IL-10 and IFNγ remain unclear.

Alongside promoting Th1 responses and IL-10 production, IL-27 also has inhibitory effects on other subsets of CD4+ T cells and cytokine production. In the setting of infections that drive Type 2 immunity, IL-27 negatively regulates Th2 cells; IL-27 receptor KO mice have enhanced Th2 responses (Bancroft et al., 2004; Lucas et al., 2003). Furthermore, it has become clear that IL-27 is a potent inhibitor of IL-17 production (Anderson et al., 2009; Murugaiyan et al., 2009) and Th17 cell differentiation (Diveu et al., 2009; Stumhofer et al., 2006), in part due to STAT1/STAT3 and T-bet signalling reducing the expression of RORγt. The anti-inflammatory properties of IL-27 also involve the suppression of IL-2 expression by Th1 cells, which in turn reduces T cell growth and survival (Owaki et al., 2006; Villarino et al., 2006; Wojno et al., 2011). Therefore, alongside promoting Th1 development and IFNγ production, IL-27 limits production of IL-2, inhibits Th2 and Th17 responses and induces IL-10, which can feedback to limit Th1 cells. IL-27 has been shown to induce a robust subset of Th1 cells that produce increased levels of IFNγ and IL-10. However, the mechanisms resulting in enhanced IL-10 production are not fully understood and therefore we were interested in comparing Th1 cells driven in the absence or presence of IL-27 by RNA-Seq to investigate the potential transcriptional mechanisms and factors that regulate IL-10 in Th1 cells.

We want to apply the method devised in Chapter 4 to perform RNA-Seq analysis on Th1 cells cultured in the presence or absence of IL-27. By analysing the transcriptional profiles of the bulk Th1 and Th1 + IL-27 cells using RNA-Seq, we hope to address the effect of IL-27 on the transcriptional profile of Th1 cells and their expression of IL-10.

5.2 (A) Results

5.2.1 IL-10 and IFNγ production by Th1 cells increases upon culture with IL-27

In Th1 cells, IL-12, alongside high antigen dose presented by APCs, is required for maximal IL-10 expression (Saraiva et al., 2009). Evidence also highlights a role for IL-27 in driving high levels of IL-10 expression in Th1 cells (Batten et al., 2008; Stumhofer et al., 2007; Zhu et al., 2015). Therefore we investigated the role of IL-27 in driving IL-10 production during *in vitro* differentiation of Th1 cells, cultured with IL-12 in the absence of APCs in the presence or absence of IL-27. Naïve CD4+CD62L+CD44loCD25- T cells were stimulated with anti-CD3 and anti-CD28, and cultured for 7 days in the presence of IL-12 and anti-IL-4 (referred to as 'Th1'), or in the presence of IL-12, anti-IL-4 and IL-27 (referred to as 'Th1 + IL-27'). The addition of IL-27 did not affect the minimal production of IL-2, and the production of IL-4 or IL-17 remained undetectable (**Figure 5A.1 A**), however IL-27 led to a significant increase in both IL-10 and IFNγ protein concentrations (**Figure 5A.1 A&B**).

5.2.2 Quality control analysis of bulk Th1 and Th1 + IL-27 RNA-Seq samples

5.2.2.1 Determining the optimal lower cut-off of reads

Prior to the identification of significantly differentially expressed genes between the bulk Th1 and Th1 + IL-27 samples, noise removal and quality control were carried out to ensure the integrity of the replicates. Once the RNA-Seq was performed, the transcripts were aligned, noise was removed (**Figure 5A.2 A**) and a quality control analysis was conducted to ensure the robustness of the experiments (**Figure 5A.2 B**). All the bulk Th1 and Th1 + IL-27 samples were pooled and the upper cut-off of reads for expression threshold was set at 1027711.75 reads (where at least 1 out of 6 samples had values within the cut-off), which was the maximal number of reads found for any gene. The number of entities passing expression thresholds with different lower-cut offs was assessed to determine where to set the lower cut-off (**Figure 5A.2 A**). The curve began to flatten at around 20 reads. With lower cut-offs greater than 20 the number of entities removed was minimal, suggesting background noise was mostly removed with a lower cut-off of 20. Therefore to ensure background noise was removed, but not to lose

important data, the lower cut-off was set at 20 reads. Next we pooled the data from the three biological replicates, and if any one gene in the three had a read count of above 20 then the entity would be included in analysis. With the lower cut-off set at 20, the number of entities passing through each repeat was calculated (**Figure 5A.2 B**). The results revealed that each of the replicates expressed around 12,000 entities. This showed that all of the replicates were comparable and were robust. Furthermore, as expected, we found that the mRNA expression profile of the cytokines *Il10* and *Ifng* mirrored the protein production profiles of the cells (**Figure 5A.2 C**). The Th1 + IL-27 cells expressed considerably more *Il10* and *Ifng* mRNA as compared to the Th1 cells.

5.2.2.2 PCA and cluster analysis of bulk Th1 and Th1 + IL-27 cells

We performed a principle component analysis (PCA) on the bulk Th1 and Th1 + IL-27 samples to further understand the variations in gene expression between the samples (**Figure 5A.3 A**). PCA is a technique that reduces the number of dimensions in the dataset to identify patterns. The biological replicates of the bulk Th1 and Th1 + IL-27 subsets clustered away from each other. There was greater inter-repeat variability in the bulk Th1 + IL-27 subset than between the bulk Th1 subset replicates, however there were no obvious outliers in the data with the three replicates of each subpopulation clustering together.

To further determine the robustness of the samples and understand the variations in gene expression between the samples we performed unsupervised hierarchical clustering on the conditions. Hierarchical clustering constructs a dendrogram in which entities are represented in a relationship tree that allows the visualisation of the data within one heat map. This groups the samples based on their similarity, and therefore similar samples should cluster together within the heat map. Gene expression is shown as a red-blue heat map, with red indicating upregulation, blue indicating downregulation and yellow no change. This clustering was performed on all 12,502 genes found within the samples (**Figure 5A.3 B**). As with the PCA, the dendrogram showed that the Th1 and Th1 + IL-27 subset replicates clustered away from each other, suggesting that IL-27 has a distinct impact on the gene expression of Th1 cells. From this analysis we were assured that the replicates were robust and there were no outliers. Therefore from this point forward, data from the three replicates was pooled. After pooling the data from the

replicates we ran unsupervised hierarchical clustering on conditions and found that the bulk Th1 and bulk Th1 + IL-27 subsets had visible differences in expression (**Figure 5A.3 C)**, however, overall their gene expression profiles looked very similar (as seen by the predominance of yellow).

5.2.3 Hierarchical clustering separates the genes within the bulk Th1 and Th2 + IL-27 cells into groups based on their expression profiles

Our data thus far demonstrates that culture of Th1 cells with IL-27 alters the transcriptional profile of Th1 cells. To further investigate the differential gene expression between the samples we applied statistical filtering to retain genes that were at least 3-fold up- or downregulated in at least one of the samples (repeat data pooled) vs. the baseline (median of all the samples). This resulted in a list of 307 genes that we then subjected to hierarchical clustering on entities and conditions (**Figure 5A.4.1 A**). The 307 gene set separated into two branches; 284 that were downregulated in the Th1 cells cultured in the presence of IL-27 (**Table 5A.4.1**), and 23 that were upregulated in the Th1 cells cultured in the presence of IL-27 (**Table 5A.4.2**). Therefore, culture with IL-27 seemed to predominantly result in the downregulation of gene expression (**Figure 5A.4.1 A**).

To further investigate the genes in these two groups we assessed if they were significantly associated with any GO terms or IPA pathways. The genes in **Group 1** (**Table 5A.4.1**) were related to binding, T cell differentiation, CD28 signalling in Th cells and NFAT signalling (**Figure 5A.4.1. B**). Genes of interest included the steroid metabolism (Th2 associated) gene *Cyp11a1* (Mahata et al., 2014); the glutamate receptor *Gria3*; the cell surface signalling molecule *Cd80*; *Dgkk,* which encodes the diacylglycerol kinase kappa, which is involved in the metabolism of the TCR signalling molecule DAG; *Gzmc,* which encodes the serine protease granzyme C; and the IFNγ receptor 2 (*Ifngr2*) (**Figure 5A.4.1. B**). Network analysis of these genes suggested they fall into two major interaction groups, with genes of interest falling into both groups (**Figure 5A.4.2**).

The genes in **Group 2** (**Table 5A.4.2**) were revealed to have no significant GO terms associated with them, however, IPA found immune cell communication and interferon

signalling pathways within the gene set (**Figure 5A.4.1 C**). The presence of the chemokine *Ccl9* and the interferon inducible gene *Ifit1* support these two pathways, respectively. However, of interest, the AhR signalling molecule *Arnt2* was also upregulated in Th1 cells cultured in the presence of IL-27 (**Figure 5A.4.1 C**). No interaction networks were found between the genes in this set.

In conclusion, though there were significant differences between Th1 cells cultured in the presence or absence of IL-27, these were not dramatic. The upregulation of interferon signalling pathways is in conjunction with IL-27 increasing IFNγ production by these cells, and furthermore IFNγ is known to feedback on the IFNγR2 to downregulate its expression (Bach et al., 1995). However, this analysis revealed no other substantial effects of IL-27 on networks and pathways within Th1 cells. We were aware that many factors involved in IL-27 driven Th1 cell differentiation and IL-10 production may be undetectable due to masking by the large IFNγ- IL-10- subpopulation of cells within the bulk cultures. Therefore we wanted to assess whether the separation of different intracellular cytokine producing subpopulations with the heterogeneous Th1 and Th1 + IL-27 bulk populations could reveal additional novel factors involved in IL-27 driven Th1 cell differentiation and IL-10 production.

Chapter 5 (B) In depth analysis of the different intracellular cytokine producing subpopulations within these Th1 and Th1 + IL-27 subsets

We next asked the question whether the separation of subpopulations within the heterogeneous Th1 and Th1 + IL-27 bulk populations could reveal additional novel factors involved in IL-27 driven Th1 cell differentiation and IL-10 production; which may be undetectable due to the potential masking by the large IFNγ- IL-10- subpopulation of cells within the bulk cultures.

5.3 (B) Background

Throughout this investigation, and in the literature, it has been shown that high levels of heterogeneity exist within all Th cell subsets (O'Garra et al., 2011; Zhu et al., 2010). Even in very similar cell types, gene expression has been shown to be very heterogeneous and suggested to be stochastic (Huang, 2009). Within an individual Th subset, there are multiple subpopulations of cells that can be distinguished by their patterns of intracellular cytokine production. *In vitro* differentiated Th1 cells can produce IFNγ alone, or IFNγ together with IL-10, but also maintain a population of cells that do not express either IFNγ or IL-10. It is increasingly understood that genes associated with subpopulations of cells within a heterogeneous population may not be revealed unless these cells are separated from the bulk population (Mahata et al., 2014) (Kuchroo VK, International Congress of Immunology, 2013). Therefore, it is hard to draw conclusions about the regulation of an individual cytokine within these heterogeneous populations, as not all cells within that subset will express that cytokine. Furthermore, in our bulk Th1 and Th1 + IL-27 populations at least half of the cells do not express IFNγ or IL-10 and therefore the RNA-Seq results may be biased by this major IFNγ- IL-10- subpopulation over the IFNγ+ IL-10- and IFNγ+ IL-10+ subpopulations.

With the protocol developed in Chapter 4 we can now perform RNA-Seq analysis on different intracellular cytokine producing subpopulations within the Th1 and Th1 + IL-

27 subsets, to try and shed light on the mechanisms behind IL-10 regulation in these cells. By comparing these results to those obtained from analysis of bulk Th1 populations driven similarly we hope to determine additional information that may be gleaned from analysis of the separated cytokine producing subpopulations. Therefore our question was, could separation of different subpopulations within heterogeneous Th1 bulk populations reveal additional novel factors involved in IL-27 driven Th1 differentiation and IL-10 production?

5.4 (B) Results

5.4.1 Separating different intracellular cytokine producing subpopulations of Th1 cells

As highlighted in **Figure 5A.1**, the Th1 and Th1 + IL-27 subsets are heterogeneous populations of cells with regard to protein production. Therefore we were interested in applying the technique developed in Chapter 4 to separate the different intracellular cytokine producing subpopulations within the Th1 and Th1 + IL-27 subsets. We elected to carry out RNA-Seq analysis on the Th1 and Th1 + IL-27 subsets as we were interested in genes affected by IL-27 and genes associated with the different intracellular cytokine producing subpopulations. We separated these subsets into different intracellular cytokine producing subpopulations based on the production of IFNγ and IL-10 (**Figure 5B.1**). mRNA from three different subpopulations: IFNγ- IL-10-, IFNγ+ IL-10- and IFNγ+ IL-10+, was extracted for RNA-Seq analysis.

The separated subpopulations represented similar percentages of the bulk live CD4+ Th1 and Th1 + IL-27 populations in the three repeat experiments (**Figure 5B.1**). In the Th1 subset, the IFNγ- IL-10- subpopulations represented on average 62% of the bulk, the IFNγ+ IL-10- subpopulations 27.4%, and the IFNγ+ IL-10+ subpopulations 3.9%. Therefore the cytokine producing subpopulations consisted of about 32% of the bulk population (**Figure 5B.1 A**), although the IL-10 producing subpopulation was the lowest. In the Th1 + IL-27 subset, the IFNγ- IL-10- subpopulations represented on average 44.5% of the bulk, the IFNγ+ IL-10- subpopulations 33.4%, and the IFNγ+ IL-10+ subpopulations 11.4%. Therefore the cytokine producing subpopulations consisted of about 45% of the bulk population (**Figure 5B.1 B**). The IFNγ+ IL-10- and IFNγ+ IL-10+ subpopulations both showed an increase in percentage as a result of the addition of IL-27.

5.4.2 Quality control analysis of Th1 and Th1 + IL-27 subpopulation RNA-Seq samples

5.4.2.1 Determining the optimal lower cut-off of reads

As with the analysis of the bulk Th1 and Th1 + IL-27 samples we performed noise removal and quality control to ensure the integrity of the replicates. Once the RNA-Seq was performed, the transcripts were aligned, noise was removed (**Figure 5B.2 A**) and a quality control analysis was conducted to ensure the robustness of the experiments (**Figure 5B.2 B**). All the Th1 and Th1 + IL-27 samples were pooled and the upper cut-off of reads for expression threshold was set at 1165880.5 reads (where at least 1 out of 24 samples had values within the cut-off), which was the maximal number of reads found for any gene. The number of entities passing expression thresholds with different lower-cut offs was assessed to determine where to set the lower cut-off (**Figure 5B.2 A**). Again, the curve began to flatten at around 20 reads and the lower cut-off was set at 20 reads. Next we pooled the data from the three biological replicates of each subpopulation, and if any one gene in the three had a read count of above 20 then the entity would be included in the analysis. With the lower cut-off set at 20, the number of entities passing through each repeat was calculated (**Figure 5B.2 B**). The results revealed that each of the replicates expressed between 10,000 – 12,000 entities. This showed that all of the replicates are comparable and are robust.

5.4.2.2 PCA and cluster analysis of Th1 and Th1 + IL-27 subpopulations

We performed PCA on the Th1 and Th1 + IL-27 subpopulations to further understand the variations in gene expression between the samples (**Figure 5B.3 A**). As with the analysis of the bulk unseparated populations earlier, the Th1 and Th1 + IL-27 subsets clustered away from each other. Within both subsets, the cytokine producing subpopulations, IFNγ+ IL-10- and IFNγ+ IL-10+, clustered separately from the non-cytokine producing subpopulation, IFNγ- IL-10-. As expected the bulk Th1 and Th1 + IL-27 cells fell between the cytokine producing and non-producing subpopulations (**Figure 5B.3 A**). Again there was greater inter-repeat variability between the samples of the Th1 + IL-27 subset than between the samples of the Th1 subset, however there

were no obvious outliers in the data with the three replicates of each subpopulation clustering together.

To further determine the robustness of the samples and understand the variations in gene expression between the samples we performed unsupervised hierarchical clustering on the conditions. This clustering was performed on all 12,114 genes found within the samples (**Figure 5B.3 B**). As with the PCA, the dendrogram showed that the Th1 and Th1 + IL-27 subsets clustered away from each other, suggesting that IL-27 has a distinct impact on the gene expression of Th1 cells. The cytokine producing subpopulations, IFNγ+ IL-10- and IFNγ+ IL-10+, cluster separately from the bulk cells and non-cytokine producing subpopulations, IFNγ- IL-10- (**Figure 5B.3 B**), suggesting the production of cytokines also has distinct effects on gene expression. From this analysis we were assured that the replicates were robust and there were no outliers. Therefore from this point forward, data from the three replicates was pooled.

5.4.2.3 Different intracellular cytokine producing subpopulations within the Th1 and Th1 + IL-27 have different transcriptional profiles

After pooling the data from the replicates we again ran unsupervised hierarchical clustering on conditions, and found that the major factor separating the subpopulations was the presence or absence of IL-27 in the culture (**Figure 5B.4 A**). Furthermore, these differences could not be attributed to differences in the expression of the Th1 master regulator *Tbx21*; as all of the subpopulations in both the Th1 and Th1 + IL-27 subsets expressed similar levels of this gene (**Figure 5B.4 B**). Again, within the Th1 and Th1 + IL-27 subsets the IFNγ+ IL-10- and IFNγ+ IL-10+ subpopulations clustered together, and separately from the bulk and IFNγ- IL-10- subpopulations. This suggested, that even though in the Th1 + IL-27 subset, where the cytokine producing and non-cytokine producing subpopulations represented equal proportions of the bulk cells, the gene expression profile of the bulk population mirrored that of the IFNγ- IL-10- subpopulation. This further suggests that the detection of genes expressed at a lower level may be limited.

5.4.3 Hierarchical clustering separates the Th1 and Th1 + IL-27 samples into groups based on their gene expression profiles

Our data thus far demonstrates that culture of Th1 cells with IL-27, or the production of cytokines within our Th1 or Th1 + IL-27 cultured cells, alters the transcriptional profile of Th1 cells. To further investigate the differential gene expression between the samples we applied statistical filtering to retain genes that were at least 3-fold up- or downregulated in at least one of the samples (repeat data pooled) vs. the baseline (median of all the samples). This resulted in a list of 1944 genes that we then subjected to hierarchical clustering on entities and conditions (**Figure 5B.5**). This was dramatically more genes than the 307 genes found in the analysis of the bulk unseparated populations (**Figure 5A.4.1**).

The 1944 gene set separated into two branches; the cytokine producers clustered into one group and the IFNγ- IL-10- subpopulations clustered with the bulk un-separated cells. Within these two clusters, the Th1 subpopulations clustered together and the Th1 + IL-27 subpopulations clustered together. However, there were clear groups of genes with different transcriptional profiles within this 1944 set of genes. Genes within this set could be separated into 6 groups. Groups of genes were manually curated based on the dendrogram and experimental hypotheses. Four groups of genes had altered expression resulting from the presence or absence of IL-27; three groups of genes had altered expression in the different cytokine producing subpopulations.

Groups of genes that are affected by the presence of IL-27 in the culture:

- Group 1: 146 genes downregulated in Th1 cells cultured with IL-27, regardless of cytokine production.
- Group 2: 349 genes downregulated in Th1 cells cultured with IL-27. Most pronounced in the IFNγ- IL-10- subpopulation.
- Group 3: 299 genes downregulated in Th1 cells cultured with IL-27. More pronounced in the cytokine producing subpopulations.
- Group 4: 86 genes upregulated in Th1 cells cultured with IL-27.

Groups of genes that correlated with the production of cytokines:

- Group 5: 586 genes downregulated in cytokine producing subpopulations.
- Group 6: 37 genes upregulated in cytokine producing subpopulations. More pronounced in the IFNγ+ IL-10- subpopulation
- Group 7: 124 genes upregulated in cytokine producing subpopulations. More pronounced in the IFNγ+ IL-10+ subpopulations.

5.4.3.1 Groups of genes that are affected by the presence of IL-27 in the culture

5.4.3.1.1 Three groups of genes were downregulated in Th1 cells cultured with IL-27

Group 1 consisted of 146 genes (**Table 5B.6.1**) that were downregulated in Th1 cells cultured with IL-27, regardless of cytokine production (**Figure 5B.6.1 A&B**). Pathways and terms related to this list of genes included cell projection, tryptophan degradation and glutathione redox reactions (**Figure 5B.6.1 C**). The genes for two glutamate receptors were included in this list, *Gria3* and *Grin1*. The list also contained *Dgkk*, which encodes the diacylglycerol kinase kappa, which is involved in the metabolism of the TCR signalling molecule DAG. Furthermore, *Aldh3a1*, which encodes the aldehyde dehydrogenase 3 family member A1 and is involved in the AhR pathway and metabolism, was present in this group of genes. The actual read number of most of the genes within this group were relatively low, as highlighted by the raw read number of the genes *Gria3*, *Grin1* and *Dgkk*, which are all below 80 (**Figure 5B.6.1 D**). The expression of *Gria3* and *Grin1* followed a similar pattern, being more highly expressed in the bulk and all of the subpopulations within the Th1 subset compared to the Th1 + IL-27 subset. However, *Dgkk* had a more complex pattern of expression. It was highly downregulated in the bulk Th1 subset compared to Th1 + IL-27, and in the IFNγ- IL-10- and IFNγ+ IL-10- subpopulations within the Th1 subset compared to Th1 + IL-27. However, both the Th1 and Th1 + IL-27 IFNγ+ IL-10+ subpopulations had very low levels of *Dgkk* expression regardless of the presence of IL-27. This suggests *Dgkk* was downregulated in cells cultured in the presence of IL-27 and in cells that produce IL-10 regardless of the presence of IL-27. To further elucidate the interaction of genes within this group we performed network analysis, and found that most of the genes did not form direct or indirect interactions with one another, however the main network

involved the two glutamate receptors GRIA3 and GRIN1 (**Figure 5B.6.2**). MAPK13, the p38 MAP kinase 13, which is a positive regulator of *Il10* expression in macrophages (Chi et al., 2006; Hammer et al., 2006), and LTBR, the lymphotoxin β receptor, were also involved in a small signalling network within this group of genes (**Figure 5B.6.2**). 95 of the genes in this list (**Table 5B.6.1** highlighted in blue) were also seen to be downregulated by IL-27 in the analysis of the bulk unseparated Th1 and Th1 + IL-27 cells (Group 1, **Table 5A.4.1**).

Group 2 consisted of 349 genes (**Table 5B.7.1**) that were downregulated in Th1 cells cultured with IL-27. This downregulation was most pronounced in the IFNγ- IL-10- subpopulation (**Figure 5B.7.1 A&B**), showing that even though these cells did not upregulated cytokine production they had received signals from IL-27. Pathways and terms related to this list of genes included cell-substrate junction assembly, positive regulation of metabolism, FXR/RXR activation and RhoGDI signalling (**Figure 5B.7.1 C**). The FXR/RXR signalling pathway is involved in lipid metabolism, and heterodimers of retinoid X receptors (RXR) with PPARγ act together to regulate gene expression (Keller et al., 1993). Supporting this was the presence of the gene *Pparg* in this group, suggesting certain forms of metabolism are downregulated by IL-27 in Th1 cells. Genes in this group included the cytokine *Il6*, the transcription factors *Eomes* and *Pparg*, the enzyme *Cyp11a1*, the chemokine ligand *Cxcl3* and the cell surface signalling molecule *Cd80* (**Figure 5B.7.1 C**) *Pparg* and *Cd80* were both downregulated by IL-27, and this was seen in the analysis of the bulk unseparated Th1 and Th1 + IL-27 cells and in all of the Th1 subpopulations. *Cyp11a1* and *Eomes*, the downregulation of which was not seen in the analysis of the bulk unseparated Th1 and Th1 + IL-27 cells, were more highly expressed in the cytokine producing subpopulations in the Th1 subset compared to the IFNγ- IL-10- subpopulation (**Figure 5B.7.1 D**).

Of note the raw read numbers of the genes within **Group 2** were much higher than those in **Group 1** (**Figure 5B.6.1 D & 5B.7.1 D**). Network analysis of the genes in **Group 2** revealed that *Il6* was a core node in the signalling interactions within this group of genes (**Figure 5B.7.2**). As seen in **Figure 5B.7.2** IL-6 interacts directly with PPARγ, which is upstream of CYP11a1. IL-6 and CD80 also formed a triangle of direct and indirect interactions (**Figure 5B.7.2**). However, the raw reads of *Il6* expression were extremely low and therefore the physiological importance of this cytokine in these

findings is uncertain. 53 of the genes in this list (**Table 5B.7.1** highlighted in blue) were also downregulated by IL-27 in the analysis of the bulk unseparated Th1 and Th1 + IL-27 cells (Group 1, **Table 5A.4.1**).

Group 3 consisted of 299 (**Table 5B.8.1)** genes that were downregulated in Th1 cells cultured with IL-27. This was more pronounced in the IFNγ+ IL-10- and IFNγ+ IL-10+ subpopulations (**Figure 5B.8.1 A&B**). There were no significant GO terms associated with this set of genes. Pathways related to this list of genes included NFAT signalling, AhR signalling and tryptophan degradation (**Figure 5B.8.1 C**). NFAT signalling was also found to be downregulated by IL-27 in the bulk Th1 and Th1 + IL-27 comparison. We showed earlier that the culture of Th1 cells with IL-27 resulted in the downregulation of *Ifngr2* in the analysis of the bulk unseparated Th1 and Th1 + IL-27 cells (**Figure 5A.4.1**). This downregulation of *Ifngr2* was also seen in all of the Th1 + IL-27 subpopulations (**Figure 5B.8.1 D**). However, regardless of IL-27 the IFNγ+ IL-10- and IFNγ+ IL-10+ subpopulations showed markedly reduced *Ifngr2* expression as compared to the IFNγ- IL-10- subpopulations (**Figure 5B.8.1 D**). This suggests that both IL-27 and cytokine production can result in the downregulation of *Ifngr2* expression, although it is unclear whether the latter is via a cell intrinsic mechanism.

Other genes in this **Group 3** included the aldehyde dehydrogenases *Aldh1l1* and *Aldh5a1*, the induction of which may be related to AhR signalling (Elizondo et al., 2000), the Indoleamine-pyrrole 2,3-dioxygenase gene *Ido1* that is involved in tryptophan degradation and the androgen receptor *Ar* (**Figure 5B.8.1 C**). Analysis of the raw read number of *Aldh5a1* revealed that the Th1 and Th1 + IL-27 subpopulations express similar amounts of this gene, and IL-27 only results in its downregulation in the cytokine producing subpopulations (**Figure 5B.8.1 D**). However, the raw read number of *Aldh5a1* also highlight that it is expressed to a very low level and these minor changes in expression may not be physiologically relevant. The only significant interaction network within this set of genes stemmed from the androgen receptor (**Figure 5B.8.2**). Only 32 of the genes in this list (**Table 5B.8.1** highlighted in blue) were also downregulated by IL-27 in the analysis of the bulk unseparated Th1 and Th1 + IL-27 cells (Group 1, **Table 5A.4.1**).

5.4.3.1.2 One group of genes was upregulated in Th1 cells cultured with IL-27

Group 4 consisted of 86 genes (**Figure 5B.9.1**) that were upregulated in Th1 cells cultured with IL-27. Some of these genes were upregulated in all of the subpopulations; IFNγ- IL-10-, IFNγ+ IL-10- and IFNγ+ IL-10+, within the Th1 + IL-27 subset, whereas genes were only upregulated in the cytokine producing subpopulations; IFNγ+ IL-10- and IFNγ+ IL-10+, within the Th1 + IL-27 subset (**Figure 5B.9.1**). We showed earlier that the culture of Th1 cells with IL-27 resulted in the upregulation of *Arnt2* in the analysis of the bulk unseparated Th1 and Th1 + IL-27 cells (**Figure 5A.4.1**). This downregulation of *Arnt2* was also seen in all of the Th1 + IL-27 subpopulations (**Figure 5B.9.1 A**). However, other genes of interest not revealed in the analysis of the bulk unseparated Th1 and Th1 + IL-27 cells included: *Maf,* which encodes the transcription factor c-Maf, *Nfkbid,* which encodes the NFκB inhibitor delta and *Tnf*. *Maf* and *Arnt2* were by IL-27 upregulated in all of the subpopulations; IFNγ- IL-10-, IFNγ+ IL-10- and IFNγ+ IL-10+ (**Figure 5B.9.1 A&D**). *Nfkbid* and *Tnf* were only upregulated by IL-27 in the cytokine producing subpopulations; IFNγ+ IL-10- and IFNγ+ IL-10+ (**Figure 5B.9.1 A&D**). TNF, which can be produced by Th1 cells (Cherwinski et al., 1987), and is generally associated with negative regulation of IL-10 (Evans et al., 2014), was a central node in the interactions between MAF, ARNT2 and other genes within this group (**Figure 5B.9.2**). Therefore, alongside IL-10 and IFNγ, IL-27 appears to drive increased expression of *Tnf* in Th1 cells. ID3 was involved in many chemokine-signalling pathways and interacted with Maf (**Figure 5B.9.2**). 13 of the genes in this list (**Table 5B.9.1** highlighted in red) were also found to be upregulated by IL-27 in the analysis of the bulk unseparated Th1 and Th1 + IL-27 cells (Group 2, **Table 5A.4.2**).

5.4.3.2 Groups of genes that correlated with the production of cytokines

5.4.3.2.1 One group of genes was downregulated in cytokine producing subpopulations

Group 5 consisted of 586 genes (**Table 5B.10.1**) that were downregulated in cytokine producing subpopulations. However this was not irrespective of IL-27. The genes in this group were most highly downregulated in the Th1 + IL-27 cytokine producing subpopulations; IFNγ+ IL-10- and IFNγ+ IL-10+, and most highly upregulated in the

Th1 + IL-27 non-cytokine producing subpopulation; IFNγ- IL-10- (**Figure 5B.10.1 A&B**). Therefore the difference in expression of these genes between the cytokine producing and non-cytokine producing subpopulations was more pronounced in the presence of IL-27. 6 of the genes in this list (**Table 5B.10.1** highlighted in red) were also upregulated by IL-27 in the analysis of the bulk unseparated Th1 and Th1 + IL-27 cells (Group 2, **Table 5A.4.2**), this is in conjunction with the observation that the upregulation of these genes was not irrespective of IL-27.

Pathways and terms related to **Group 5** of genes were mostly related to interferon signalling (**Figure 5B.10.1 C**), and additionally many interferon related genes were present in this group (**Table 5B.10.1**). STAT1, which regulates genes downstream of the interferon receptors including IFNγR1 (Darnell et al., 1994), was present in this list. Multiple *Gbp* genes, which are known to be IFNγ responsive (Shenoy et al., 2007), were also present, as well as *Irf7* and *Ifit2*, which are also interferon inducible (Levy et al., 1986; Nguyen et al., 1997) (**Figure 5B.10.1 C**). This downregulation of interferon signalling in the different cytokine producing subpopulations seemed to be unaffected by the presence of IL-27, and upon analysis of the bulk populations there was little difference in the raw read values of *Ifngr1* and *Stat1* (**Figure 5B.10.1 D**). However, *Ifngr1* and *Stat1* expression may have been slightly upregulated in the Th1 + IL-27 IFNγ- IL-10- subpopulation compared to the Th1 IFNγ- IL-10- subpopulation (**Figure 5B.10.1 D**). Again, as seen in the heat map and profile plot, the raw read values of *Ifngr1* and *Stat1* were highly downregulated in the IFNγ+ IL-10- and IFNγ+ IL-10+ cytokine producing subpopulations of both the Th1 and Th1 + IL-27 subsets compared to the IFNγ- IL-10- subpopulations (**Figure 5B.10.1 D**). This suggests that intrinsic downregulation of the *Ifngr1* is occurring in cytokine producing cells. The central role of interferon signalling in this group of genes was highlighted by the core nodes that STAT1, IFNγR1 and IRF7 formed in the interactions between the genes in this group (**Figure 5B.10.2**). This demonstrates that analysis of heterogeneous cell populations may present apparent upregulation of genes in the bulk population, which is actually occurring in only a subpopulation of the cells.

5.4.3.2.2 Two groups of genes were upregulated in cytokine producing subpopulations

Group 6 consisted of 37 genes (**Figure 5B.11 A**) that were upregulated in cytokine producing subpopulations except surprisingly in the Th1 + IL-27 IFNγ+ IL-10+ subpopulation, suggesting an inverse correlation with IL-10 (**Figure 5B.11 A&B**). This group of genes were highly related to glutamate receptor signalling, PPAR signalling and TGFβ signalling (**Figure 5B.11 C)**. *Dusp9* was present in this list of genes, which encodes the phosphatase DUSP9 that is involved in the suppression of ERK signalling (Keyse, 2000). *Dusp9* appeared to be downregulated by IL-27 in the bulk Th1 + IL-27 subset, however when looking at the individual subpopulations this was not always the case (**Figure 5B.11 D**). The read number of *Dusp9* highlighted that this gene was upregulated in the Th1 and Th1 + IL-27 cytokine producing subpopulations, and was associated with high IFNγ production but was downregulated in IL-27 driven IFNγ+ IL-10+ cells (**Figure 5B.11 D**).

Network analysis of **Group 6** revealed two glutamate receptors, *Grm1* and *Gria1*, were present in this gene list, and the interaction between these genes was the only significant interaction found between genes in this list (**Figure 5B.11 E**). *Grm1* encodes the mGlu1R glutamine receptor that is coupled to the ERK signalling pathway (Pacheco et al., 2004) and the stimulation of this receptor has been correlated to IL-10 secretion by Th1 cells (Pacheco et al., 2006). However, the read number of *Grm1* transcripts were very low, even in the groups with increased expression. There was no overlap with genes in this list and those found to be affected by IL-27 in the analysis of the bulk unseparated Th1 and Th1 + IL-27 cells (Group 1 & 2, **Table 5A.4.1 & 2**). Therefore, we highlight many novel factors correlating with cytokine production that would not have been revealed without separating the different subpopulations.

Group 7 consisted of 124 genes (**Table 5B.12.1**) that were more highly expressed in the cytokine producing subpopulations. This was more pronounced in the IFNγ+ IL-10+ subpopulations, suggesting these genes more closely correlated with IL-10 expression than IFNγ expression (**Figure 5B.12.1 A&B**). The pathways and terms related to this group were mostly involved in immune processes including the regulation and signalling of cytokines, communication between immune cells and inflammatory responses (**Figure 5B.12.1 C).** However, genes within this list are also known to negatively regulate some metabolic processes (**Figure 5B.12.1 C**), including *Wnt6,*

Wnt9b, Wnt11, Cav1 and *Foxl2* (GO annotations). As expected genes within this list encoded many cytokines and chemokines, including *Il10, Ifng, Il3, Il1a, Il23a, Il17f, Il22, Csf2, Ccl1, Ccl3* and *Cxcl13* (**Figure 5B.12.1 C**). Of note, the expression of *Il10* and *Ifng* mRNA followed the same pattern as intracellular IL-10 and IFNγ protein production seen in the FACS plots (**Figure 5B.12.1 D**). These genes were all upregulated in the cytokine producing subpopulations compared to the IFNγ- IL-10- subpopulations, however, the patterns of expression at the raw read number varied (**Figure 5B.12.1 D**). As expected *Il10* was only upregulated in the IFNγ+ IL-10+ subpopulations, and to a greater extent in the Th1 + IL-27 cells, while *Ifng* was expressed in both the IFNγ+ IL-10- and IFNγ+ IL-10+ subpopulations; corroborating the protein levels seen by intracellular cytokine staining. *Csf2*, which encodes the cytokine GM-CSF, was upregulated in both cytokine producing subpopulations but to a greater extent in the Th1 subset, but this was not reflected when comparing the whole populations, where *Csf2* expression appeared to be comparable between the Th1 and Th1 + IL-27 subsets. *Gzma*, which encodes the serine protease granzyme A, was mostly upregulated in the Th1 IFNγ+ IL-10- subpopulation, whereas in the whole populations the Th1 + IL-27 subset appeared to express higher *Gzma* expression. Therefore, although all of these genes were associated with the cytokine producing subpopulations, they had different expression profiles. Network analysis revealed many of these cytokines at the centre of interactions between these genes (**Figure 5B.12.2**). Again was no overlap with genes in this list and those found to be affected by IL-27 in the analysis of the bulk unseparated Th1 and Th1 + IL-27 cells (Group 1 & 2, **Table 5A.4.1 & 2**).

Within these final gene lists it is possible there are genes that are associated with *Il10* regulation. Genes in **Group 6** were downregulated in IL-10 producing subpopulations, and therefore may have regulatory roles in repressing *Il10* expression. Genes in **Group 7** were upregulated in IL-10 producing subpopulations, and therefore may have regulatory roles in promoting *Il10* expression. However, in isolation we have little information with which to select viable candidates that may be novel factors involved in the regulation of *Il10*. Therefore it would be of use to set up a screen using RNAi or CRISPR-Cas to determine the effects on *Il10* gene expression. Comparison of these gene sets to genes up- or downregulated in the IL-10+ subpopulations of other Th cell subsets could enable the extraction of genes that consistently correlate with *Il10* expression.

5.5 Discussion

IL-27 has been shown to upregulate IL-10 production by Th1 cells (Batten et al., 2008; Stumhofer et al., 2007; Zhu et al., 2015). However, the effects of IL-27 on these cells at a transcriptional level are still poorly understood. Furthermore, cytokine production by Th1 cells is heterogeneous and the effects of IL-27 on these different intracellular cytokine producing subpopulations may vary. Therefore not only did we want to characterise the transcriptional changes IL-27 drives in Th1 cells, but also how the different intracellular cytokine producing subpopulations differ transcriptionally and how they respond to IL-27. In this Chapter we carried out RNA-Seq analysis on Th1 cells and Th1 cells cultured with IL-27, and on the intracellular cytokine producing subpopulations within these two subsets.

5.5.1 IL-27 drives increased IL-10 and IFNγ production by Th1 cells

Previous studies have shown that IL-27 drives increased IFNγ (Chen et al., 2000a; Lucas et al., 2003; Takeda et al., 2003) and IL-10 (Awasthi et al., 2007; Batten et al., 2008; Fitzgerald et al., 2007; Stumhofer et al., 2007) production from Th1 cells. When culturing Th1 cells *in vitro*, as optimised in Chapter 3, alongside IL-27 we also saw an increase in IFNγ and IL-10 protein production from Th1 cells. Interestingly, as discussed in Chapter 3, in the presence or absence of IL-27 it was the cells that produce high levels of IFNγ that also produce IL-10. None of the Th1 cells that were negative for IFNγ produced IL-10. Therefore IL-27 does not drive IL-10 production in the absence of IFNγ production. The expression of *Ifng* and *Il10* mRNA matched that of IFNγ and IL-10 protein production, in the bulk Th1 and Th1 + IL-27 subsets and in the different subpopulations.

We continue to consistently see an IFNγ- IL-10- subpopulation. This does not appear to be due to different levels of activation of the cells, as all of the subpopulations express similar high levels of the IL-2 receptor CD25. Therefore, is it possible this population is maintained due to homeostatic regulation within the heterogeneous subset. Evidence from cytokine secretion assays suggests that upon re-culture, IL-10 non-producing cells do not gain the ability to produce large amounts of IL-10 (Ahyi et al., 2009; Chang et al.,

2007). Furthermore, it has been proposed that individual Th1 cells have a quantitative cytokine memory and that within a heterogeneous population there are stable cytokine-producing and –nonproducing subpopulations (Helmstetter et al., 2015). However, we do not know if these IFNγ- IL-10- cells are stable or would start to produce cytokines if separated from the cytokine-producing cells and re-cultured. To separate these cells and re-culture them, ICS could not be used; an IFNγ reporter or secretion assay would have to be employed instead. This analysis has however revealed many transcriptional differences between this cytokine-nonproducing subpopulation and the cytokine-producing subpopulations.

5.5.2 Culture with IL-27 results in changes in the transcriptional profile of bulk populations of Th1 cells

Analysis of the transcriptional profiles of the bulk Th1 and Th1 + IL-27 subsets, revealed that the cells within each subset were transcriptionally very different. Clustering analysis revealed that the majority of genes whose expression was altered by IL-27 were downregulated. These predominantly repressive effects of IL-27 on Th1 gene expression have not previously been shown. Within this group of 284 genes, the expression of *Ifngr2*, which encodes the IFNγ receptor 2, was downregulated. This is supported by the literature, which proposes that *Ifngr2* is downregulated by IFNγ feeding back upon the Th1 cells (Bach et al., 1995). Other genes within this group are associated with lipid metabolism (*Cyp11a1*), glutamate signalling (*Gria3*) and TCR signalling (*Dgkk*), however these pathways were not reflected in the GO and IPA analysis. The decreased expression of *Cd80* and multiple HLA genes resulted in the IPA analysis associating multiple T cell signalling pathways to this group of IL-27 downregulated genes. A minority of genes, 23, were upregulated by IL-27. IPA analysis revealed the upregulation of Interferon signalling by IL-27. This is as expected, owing to the increased levels of IFNγ produced by Th1 + IL-27 cells. Other genes within this group are associated with AhR signalling (*Arnt2*), however this pathway was not reflected in the GO and IPA analysis. Overall the analysis of bulk Th1 and Th1 + IL-27 subsets revealed little about the results of IL-27 on the transcriptional profiles of Th1 cells. Therefore, it was hard to elucidate any mechanisms that may regulate *Il10* gene expression. It is possible that dominant subpopulations of cells within these heterogeneous bulk Th1 and Th1 + IL-27 subsets may be masking low level changes in

gene expression. Furthermore, work on single-cell transcriptomics have highlighted how looking at covariance between transcripts in individual cells can reveal regulatory circuits that may not be obvious at the population level (Shalek et al., 2014). Therefore, our question was, could we improve this analysis and reveal more about the effects of IL-27 on Th1 cells and mechanisms that may regulate *Il10* gene expression by separating the different intracellular cytokine producing subpopulations within the Th1 and Th1 + IL-27 subsets?

5.5.3 Different intracellular cytokine producing Th1 and Th1 + IL-27 subpopulations have different transcriptional profiles

Analysis of the transcriptional profiles of the bulk Th1 and Th1 + IL-27 subsets, and the different intracellular cytokine producing subpopulations within the Th1 and Th1 + IL-27 subsets, revealed that the bulk and the three different subpopulations within each subset were transcriptionally very different. We found earlier that the analysis of bulk unseparated Th1 and Th1 + IL-27 subsets revealed that the expression of 307 genes was altered by the presence of IL-27, 284 of these being downregulated by IL-27. When analysing the different subpopulations within the Th1 and Th1 + IL-27 subsets we revealed that the expression of 880 genes was altered by the presence of IL-27, 794 of these being downregulated by IL-27. Therefore by separating the heterogeneous bulk Th1 and Th1 + IL-27 subsets we have revealed many more genes affected by IL-27.

Clustering and correlative analysis suggested that the cytokine producing subpopulations within each subset were very closely related to one another, but were very different from the IFNγ- IL-10- subpopulation. Furthermore, the bulk Th1 or Th1 + IL-27 subset transcriptional signature was most similar to the IFNγ- IL-10- subpopulation. Therefore, when analysing these samples by RNA-Seq the IFNγ- IL-10- subpopulation biased the transcriptional profile of the bulk population. In the Th1 subset, the IFNγ- IL-10- subpopulation represented 62% of the culture, while the cytokine producing subpopulations represented 32%, and therefore it is not unexpected that the bulk mRNA is dominated by the IFNγ- IL-10- signature. However, in the Th1 + IL-27 subset, the IFNγ- IL-10- subpopulation represented 45% of the culture, while the cytokine producing subpopulations represented 45%, and therefore one would expect

the bulk mRNA to have a transcriptional profile half way between the IFNγ- IL-10- and cytokine producing subpopulations. Consequently, we can conclude that the transcriptional profile of a heterogeneous population of cells is not equally representative of all the subpopulations within it. Therefore, by separating the different intracellular cytokine producing subpopulations from within a Th subset we may obtain more accurate gene expression profiling of different subpopulations and additional information regarding differential gene expression.

5.5.4 IL-27 drives transcriptional changes in the different intracellular cytokine producing subpopulations within Th1 cells

Culturing Th1 cells *in vitro* with IL-27 resulted in distinct changes in both up- and downregulation of genes compared to Th1 cells cultured in the absence of IL-27. The majority, but not all, of these changes were only seen when analysing the different intracellular producing subpopulations, rather than the bulk un-separated subsets.

5.5.4.1 IL-27 alters glutamate signalling in Th1 cells

In **Group 1**, the expression of two iGluRs was downregulated by IL-27; *Gria3* encodes the APMA receptor iGlu3R, and *Grin1* encodes the NMDA receptor 1 (**Discussion Figure 5.1**). Much evidence suggests glutamate receptors play an important role in T cell immunity and development (Pacheco et al., 2007). Glutamate can interact with different types of receptors, which are separated into two main groups; the ionotropic glutamate receptors (iGluRs) and the metabotropic glutamate receptors (mGluRs). As found in the associated GO terms, iGlu3R has been shown to be involved in T cell adhesion and migration. Furthermore, it has been postulated that upon TCR stimulation, iGlu3R may impair IL-10 production (Pacheco et al., 2006). This is supported by evidence that IL-10 impairs T cell adhesion and migration (Jinquan et al., 1995; Tan et al., 1995), and iGlu3R may promote these processes by inhibiting IL-10. Therefore, a possible mechanism by which IL-27 increases *Il10* expression in Th1 cells is by downregulating the expression of iGlu3R, which may be an inhibitor of IL-10 production.

5.5.4.2 IL-27 alters signalling associated with lipid & cholesterol metabolism

Multiple genes involved in metabolic processes were downregulated in Th1 cells cultured with IL-27, including *Cyp11a1* and *Pparg* (**Discussion Figure 5.1**). In **Group 2** the expression of the enzyme Cyp11a1 was downregulated by IL-27. Cyp11a1 is involved in steroid metabolism and is a key enzyme in the conversion of cholesterol to the steroid hormone pregnenolone. Steroids are immunoregulators and have suppressive effects on inflammation (Sakiani et al., 2013). Recent publications have highlighted the specific upregulation of *Cyp11a1* in Th2 cells (Mahata et al., 2014). Therefore IL-27 appears to downregulate the expression of genes associated with Th2 cells, and genes that drive immune-suppressive pathways.

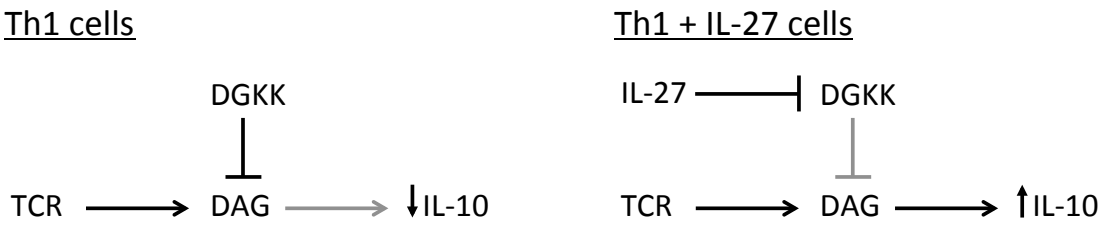
Moreover, in **Group 2** the expression of the peroxisome proliferator-activated receptor γ (PPARγ) was downregulated by IL-27. PPARγ is a nuclear hormone receptor that regulates lipid metabolism and glucose homeostasis (Waickman and Powell, 2012; Xu et al., 1999), and has also been shown to decrease IFNγ secretion in CD4+ T cells (Cunard et al., 2004). Therefore, a novel pathway by which IL-27 may regulate the increased production of IFNγ by Th1 cells may be via the downregulation of PPARγ. Furthermore, the homologue of PPARγ, PPARα has been shown to repress the transcription factors AP-1 and NF-κB (Delerive et al., 1999). It is possible that PPARγ may have similar repressive effects on these pro-inflammatory transcription factors, and therefore IL-27 may reduce this repression and in turn promote inflammatory responses along with IFNγ.

5.5.4.3 TCR signalling is altered in IL-27 driven Th1 cells

The gene *Dgkk*, which encodes diacylglycerol kinase (DGK) kappa, was downregulated by IL-27 in Th1 cells (**Group 1 & Discussion Figure 5.1**). DGKs are intracellular lipid kinases that phosphorylate diacylglycerol (DAG), enabling it to be converted to phosphatidic acid (Merida et al., 2008). This reduces DAG signalling and in turn regulates intracellular signalling. DAG is a fundamental molecule in the TCR signalling cascade, and is key in antigen receptor triggering (Cantrell, 2002). Therefore, this data suggests that IL-27 may indirectly augment TCR signalling via the downregulation of DGKs. This may explain one pathway by which IL-27 upregulates IL-10 production, as

it has been shown that increased antigen dose increases IL-10 expression by Th1 cells (Saraiva et al., 2009).

Model for postulated mechanism



The possibility that IL-27 promotes greater TCR triggering in Th1 cells is supported by the increased expression of *Nfkbid* in Th1 + IL-27 cells (**Discussion Figure 5.2**). *Nfkbid*, which encodes the NF-κB inhibitor IκBNS, was upregulated by IL-27 in **Group 3**. Induction of IκBNS expression has been correlated with TCR signal strength, with weak TCR ligands failing to trigger IκBNS expression (Fiorini et al., 2002). Furthermore, IκBNS has been associated with T cell cytokine regulation and that IκBNS deficient thymocytes and CD8+ T cells produce significantly reduced amounts of IFNγ (Touma et al., 2007). Therefore, the culture of Th1 cells with IL-27 potentially could result in increased TCR signalling, which drives *Il10* expression, and *Nfkbid* expression that in turn drives increased IFNγ production.

5.5.4.4 Molecules involved in the AhR signalling pathway are highly upregulated in the IFNγ- IL-10- subpopulation within the Th1 + IL-27 subset

Upon analysis of the bulk unseparated Th1 and Th1 + IL-27 subsets we saw a dramatic increase in the expression of *Arnt2* (**Discussion Figure 5.2**). Furthermore, analysis of the separate intracellular cytokine producing subpopulations revealed a dramatic increase in the expression of *Maf* in **Group 4** (**Discussion Figure 5.2**). Arnt2 is part of a group of proteins that are important in translocating AhR into the nucleus, where it can then act as a transcription factor (Rowlands and Gustafsson, 1997). c-Maf has been shown to promote the activation of *Il10* expression in IL-27 driven Tr1 cells (Pot et al., 2009), and IL-27 has been shown to activate AhR, which upon interaction with c-Maf transactivates *Il10* transcription (Apetoh et al., 2010). Furthermore, AhR has recently

been shown to regulate the metabolic programme that supports IL-10 producing, IL-27 driven, Tr1 cells (Mascanfroni et al., 2015). However, this has not been reported in Th1 cells. Therefore our findings are in conjunction with previous reports that IL-27 promotes c-Maf and AhR signalling.

When we characterised the expression of *Maf* and *Arnt2* in the different intracellular cytokine producing subpopulations within the Th1 + IL-27 subset we found that these factors were differentially expressed. *Maf* and *Arnt2* were most upregulated by IL-27 in the IFNγ- IL-10- subpopulation, and were expressed to a lower level in the cytokine producing subpopulations. *Maf* expression has been shown to be maximal at 72 – 96 hours of differentiation from naïve CD4+ T cells under Th1 driving conditions, after which point its expression is downregulated (Neumann et al., 2014). We show that in this heterogeneous population of Th1 cells, *Maf* expression remains high in the IFNγ- IL-10- subpopulation, and is downregulated as cytokines are produced. This perhaps points to the role of c-Maf in the regulation of cytokine expression and in turn its mechanism of action.

This data shows that IL-27 drives increased *Maf* and *Arnt2* expression, which is then downregulated upon cytokine production by the Th1 cells. It is possible that there is a cell intrinsic feedback mechanism that initiates once the cells have started to make the cytokines, which acts to reduce the induction of c-Maf and AhR. Interestingly, when returning to the raw read data, the expression of the *Il27ra* follows a similar expression profile to *Maf* and *Arnt2* (**Discussion Figure 5.2**). Therefore our data shows that in the presence of IL-27 the expression of *Maf* and *Arnt2* is upregulated in the IFNγ- IL-10- subpopulations, but upon cytokine production *Maf* and *Arnt2* expression is decreased, and this coincides with a decreased expression of *Il27ra.* Therefore, it is possible that upon the production of cytokines cells downregulate the expression of the IL-27 receptor, and therefore cannot respond further to IL-27, resulting in the signalling pathways and transcriptional networks driven by IL-27 being reduced, and in turn reducing the expression of *Maf* and *Arnt2*.

In **Group 4** the expression of *Tnf* follows the reverse expression profile to *Maf* and *Arnt2* in Th1 + IL-27 cells (**Discussion Figure 5.2**). It is under expressed in IFNγ- IL-10- subpopulations and highly expressed in the cytokine producing subpopulations

IFNγ+ IL-10- and IFNγ+ IL-10+. Corresponding with its immune effector function. It has been shown in microglia that IL-27 can downregulate TNF-α expression (Baker et al., 2010) and lymph node cells from IL-27 receptor knockout mice express significantly more TNF (Batten et al., 2006). Therefore the increase in *Tnf* expression seen in our data in the cytokine producing subpopulations of the Th1 + IL-27 subset may be because of the downregulation of the *Il27ra*.

5.5.5 Different intracellular producing subpopulations from the Th1 and Th1 + IL-27 subsets have different transcriptional profiles

Separating the Th1 and Th1 + IL-27 subsets into different intracellular cytokine producing subpopulations highlighted dramatic differences in gene expression.

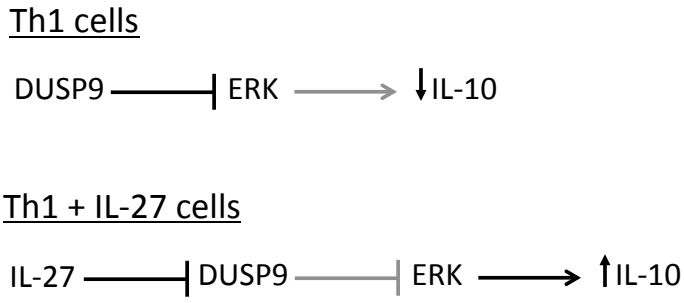
5.5.5.1 Interferon signalling is downregulated in cytokine producing subpopulations

Our data shows that in Th1 cells cultured in the presence of IL-27 the expression of *Ifngr1* and *Ifngr2* was downregulated (**Discussion Figure 5.1)**. However, irrespective of the presence of IL-27, the expression of *Ifngr1* and *Ifngr2* was also downregulated in the cytokine producing subpopulations (**Discussion Figure 5.1)**. Previous publications reveal that upon activation, Th1 cells downregulate the expression of IFNγ receptors. The expression of *Ifngr1* is downregulated in Th1 cells upon TCR engagement and signalling (Skrenta et al., 2000), while the expression of *Ifngr2* is downregulated by IFNγ feeding back upon the Th1 cells (Bach et al., 1995). Therefore in our *in vitro* culture system, we would expect all of the subpopulations within the Th1 + IL-27 subset to have downregulated the expression of both IFNγ receptors; as IL-27 drives increased IFNγ production, and as all the cells received the same dose of antigen and were in their own supernatant containing IFNγ. However, this was not that case. Therefore, these data suggest that, rather than IFNγ acting in an autocrine manner to feedback on all Th1 cells in the culture and reduce IFNγ receptor expression, there is in fact either; a cell intrinsic mechanism that results in the downregulation of IFNγ receptor expression only in cells that are expressing IFNγ; or that the IFNγ released by

the IFNγ+ cells acts in a highly local manner to mediate negative feedback only on Th1 cells producing IFNγ.

To further characterise our findings, analysis of the expression of the IFNγ receptor and interferon responsive genes in naïve CD4+ T cells and Th0 cells would be of interest. Neither naïve CD4+ T cells nor Th0 cells produce IFNγ. Naïve CD4+ T cells, which have not received TCR or IFNγ signalling, should according to the literature have maximal *Ifngr1* and *Ifngr2* expression. Th0 cells, which receive TCR signalling but are cultured in the presence of anti-IFNγ to prevent IFNγ signalling, should according to the literature have maximal *Ifngr2* but reduced *Ifngr1* expression. By comparing the naïve CD4+ T cells and Th0 cells to the Th1 subpopulations we can establish if the expression if the IFNγ receptors is downregulated by TCR or IFNγ signalling in the absence of *Ifng* expression.

5.5.5.2 DUSP9 expression is decreased in the IFNγ+ IL-10+ subpopulation within the Th1 + IL-27 subset



Dusp9 encodes the dual specificity phosphatase 9 (also known as MKP-4) (Niedzielska et al., 2015). This gene is upregulated in both the Th1 and Th1 + IL-27 IFNγ+ IL-10- subpopulations, and downregulated in the IFNγ+ IL-10+ subpopulation of the Th1 + IL-27 subset (**Group 6, Discussion Figure 5.1**). DUSP9 is a phosphatase that has an important role in the attenuation of ERK, JNK and p38 via dephosphorylation of these proteins (Muda et al., 1997). ERK signalling is also important for positive regulation of IL-10 (Saraiva et al., 2009). Furthermore, pDCs (which do not make IL-10) selectively express high levels of DUSP9 compared to cDCs (which do express IL-10) (Niedzielska et al., 2015). Therefore this recent finding, alongside our data, suggests that DUSP9 may be a negative regulator of IL-10 production via the repression of ERK signalling.

We propose that in IFNγ+ IL-10- cells *Dusp9* expression is high to enable IFNγ production in the absence of IL-10, and in IFNγ+ IL-10+ cells *Dusp9* expression is reduced to enable IFNγ and IL-10 production. The greater reduction of *Dusp9* expression in the IFNγ+ IL-10+ Th1 + IL-27 cells compared to the IFNγ+ IL-10+ Th1 cells may define one mechanism by which IL-27 drives increased IL-10 production.

5.5.5.3 Multiple genes involved in cytokine signalling are upregulated in the cytokine producing subpopulations within the Th1 and Th1 + IL-27 subsets

Group 5 was a cluster of genes that were upregulated in the cytokine producing subpopulations within the Th1 and Th1 + IL-27 subsets. Therefore, as expected *Il10* was upregulated in the IFNγ+ IL-10+ subpopulations and *Ifng* was upregulated in both the IFNγ+ IL-10- and IFNγ+ IL-10+ subpopulations. However, many other cytokines and chemokines were also upregulated in these subpopulations alongside IL-10 and IFNγ. Some of the cytokines that were upregulated alongside IL-10 and IFNγ are known to be associated with Th1 cells. The initial studies that phenotyped Th1 and Th2 clones found Th1 cells produce IL-3 and GM-CSF (Cherwinski et al., 1987). GM-CSF is made by Th1 cells in a STAT4 dependent manner (McWilliams et al., 2015) and is important in the initiation of autoimmune inflammation, particularly during EAE (Codarri et al., 2011; Herndler-Brandstetter and Flavell, 2014; Marusic et al., 2002). In support of recent publications that show IL-27 suppresses T cell GM-CSF expression (Young et al., 2012), in our data *Csf2* expression was downregulated in the Th1 + IL-27 cytokine producing subpopulations compared to the Th1 cytokine producing subpopulations. Interestingly some of the cytokines that were upregulated are not generally considered to be associated with Th1 cells, such as IL-1α and IL-23α. Furthermore, IL-22 and IL-17F, cytokines associated with Th17 cells (Awasthi and Kuchroo, 2009; Harrington et al., 2005), were upregulated in the cytokine producing subpopulations of the Th1 and Th1 + IL-27 subsets. Therefore, alongside the upregulation of IL-10 and IFNγ production in the cytokine producing subpopulations of the Th1 and Th1 + IL-27 subsets, the expression of multiple other cytokines was upregulated. It is likely that the factors and pathways driving the upregulation of *Il10* and *Ifng* expression are also acting on these other cytokine genes.

5.5.6 The identification of mechanisms that may regulate the expression of *Il10*

The analysis of the bulk unseparated Th1 and Th1 + IL-27 subsets revealed some genes and processes that were affected by IL-27, however this analysis could not be used to expose any novel genes or mechanisms that may be involved in the regulation of *Il10* gene expression, as discussed above with examples. By separating the different intracellular cytokine producing subpopulations within the Th1 and Th1 + IL-27 subsets, however, we did reveal groups of genes that were specifically downregulated or upregulated alongside IL-10. Therefore these genes could be negative or positive regulators of *Il10* expression, respectively. Nevertheless, it is difficult to be sure that these genes within these lists are involved in *Il10* regulation. These genes could have no relation of IL-10, could be regulated in parallel with IL-10, or could be regulated by IL-10. Therefore, these targets will be knocked out by CRISPR-Cas or RNAi by the O'Garra laboratory to ascertain their role in the regulation of *Il10* gene expression.

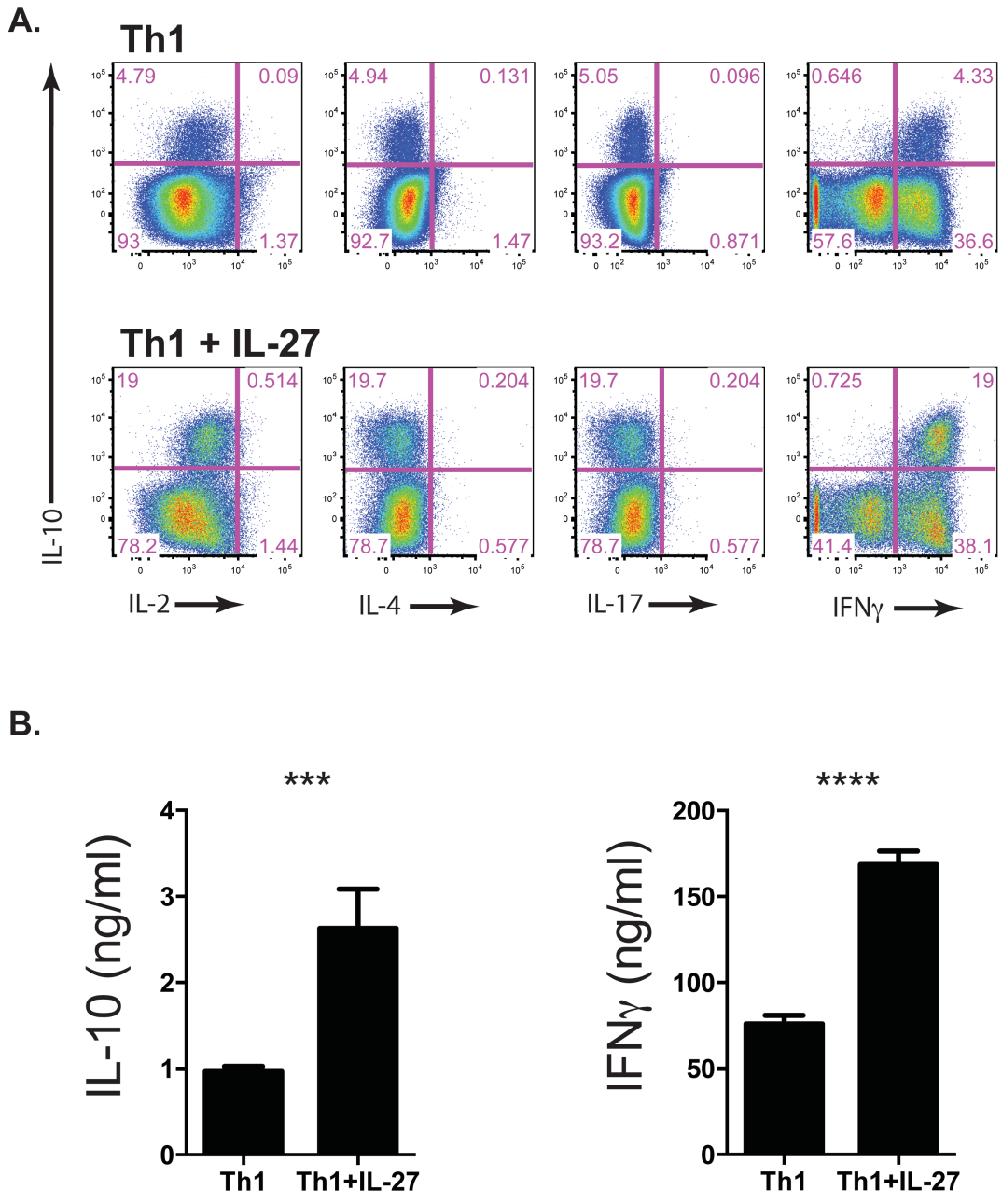


Figure 5A.1 IL-10 and IFNγ production by Th1 cells increased upon culture with IL-27

C57BL/6 naive CD4+CD62L+CD44loCD25- cells were driven with plate-bound anti-CD3 and soluble anti-CD28, and polarised to Th1 cells with IL-12 and anti-IL-4 in the presence or absence of IL-27. **A.** Plots of flow cytometric analysis of intracellular cytokine staining. Numbers show percentage of live CD4+ cells. Gating strategy described in Materials and Methods. **B.** Graphs represent cytokines in supernatants of cells determined by ELISA. Cells restimulated as described in Materials and Methods, assessed after 7 days of polarization *in vitro*. Graphs show means ± SD of triplicates, **p < 0.01, ***p < 0.001, ****p<0.0001 determined by students T-test. Representative of six experiments.

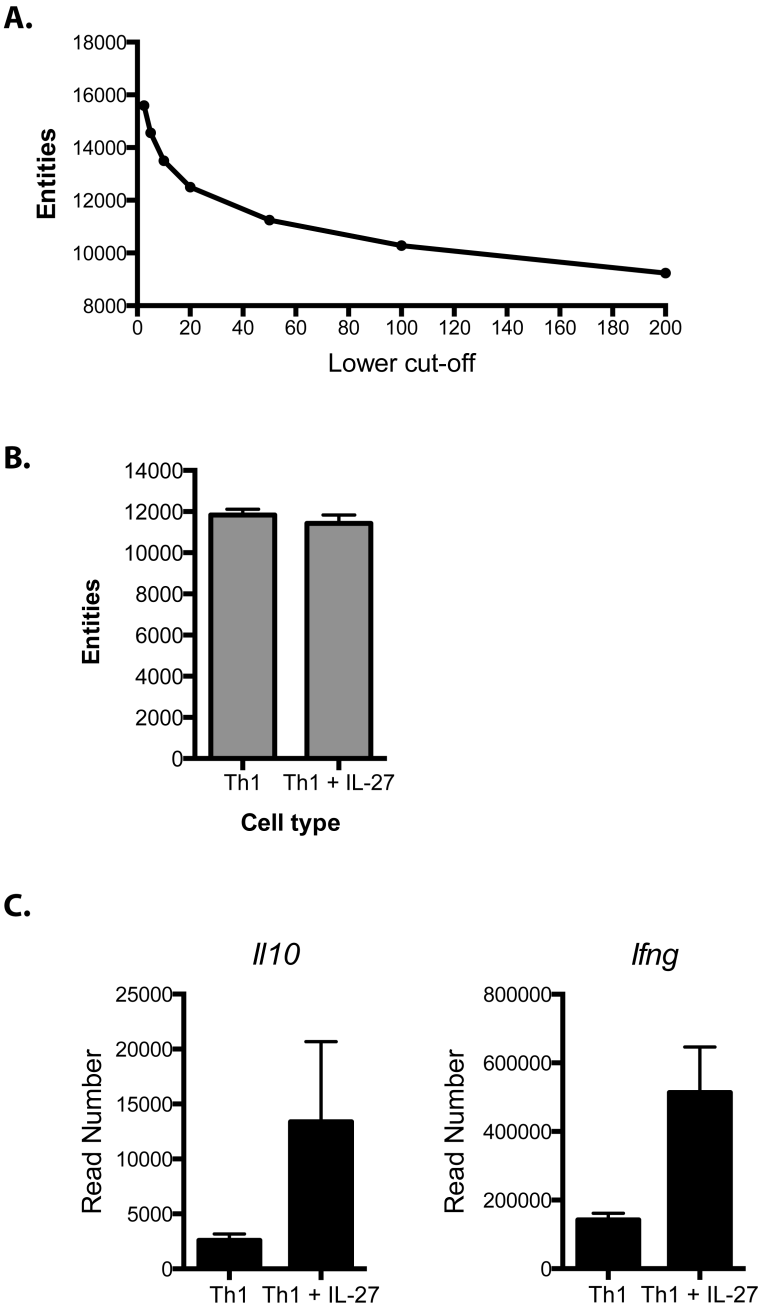


Figure 5A.2 Determining the optimal lower cut-off of reads for expression threshold of bulk Th1 and Th1 + IL-27 RNA-Seq samples

mRNA extracted and prepared for RNA-Seq as described in Materials and Methods. Using Strand NGS software reads were aligned to the transcriptome & Genome (mm10, RefSeq annotation, 95% identity, max 5% gaps, 1 read only if duplicate) and normalisation with DeSeq and no Baseline. Upper cut-off 1027711.75 (where at least 1 out of 6 samples have values within cut-off). **A.** The number of entities passing expression thresholds with indicated lower cut-offs. All bulk Th1 and Th1 + IL-27 samples were pooled for this test. **B.** Using an expression threshold with a lower cut-off of 20 reads, the number of entities in each repeat of the bulk Th1 and Th1 + IL-27 subsets was determined. **C.** Graphs of *Il10* and *Ifng* read number from the RNA-Seq data.

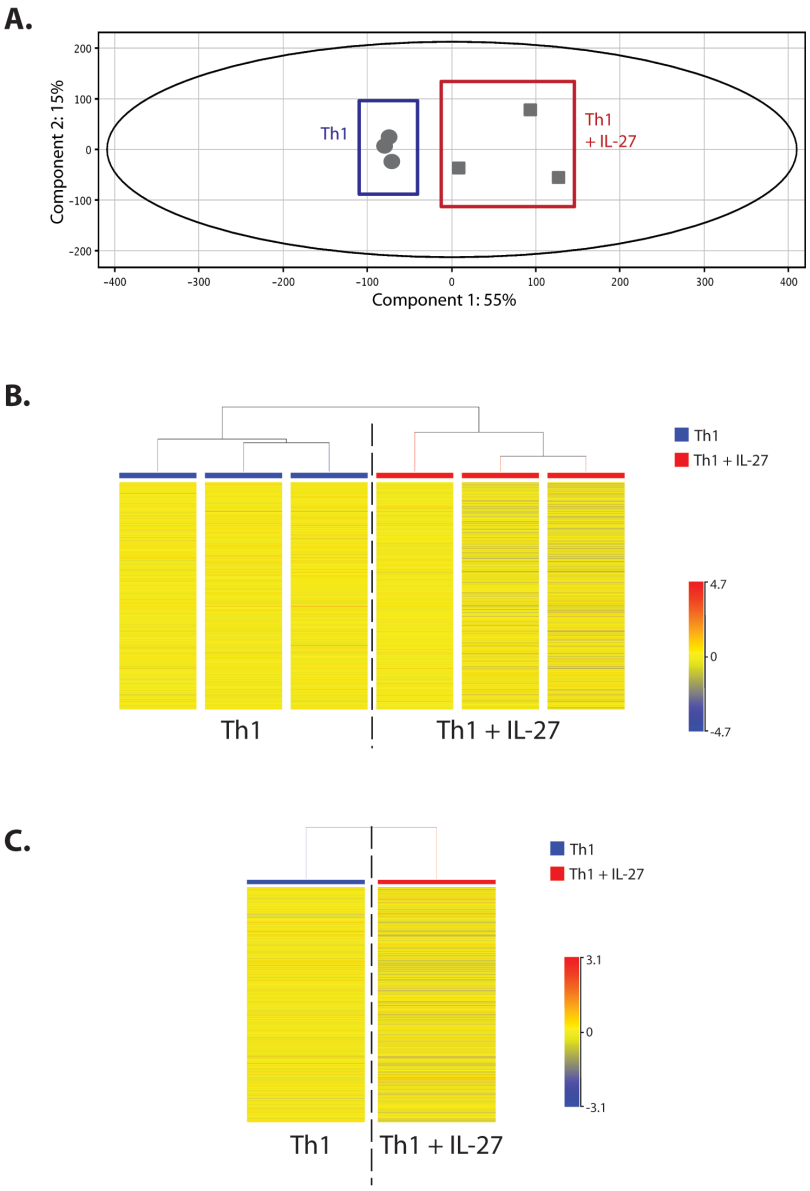
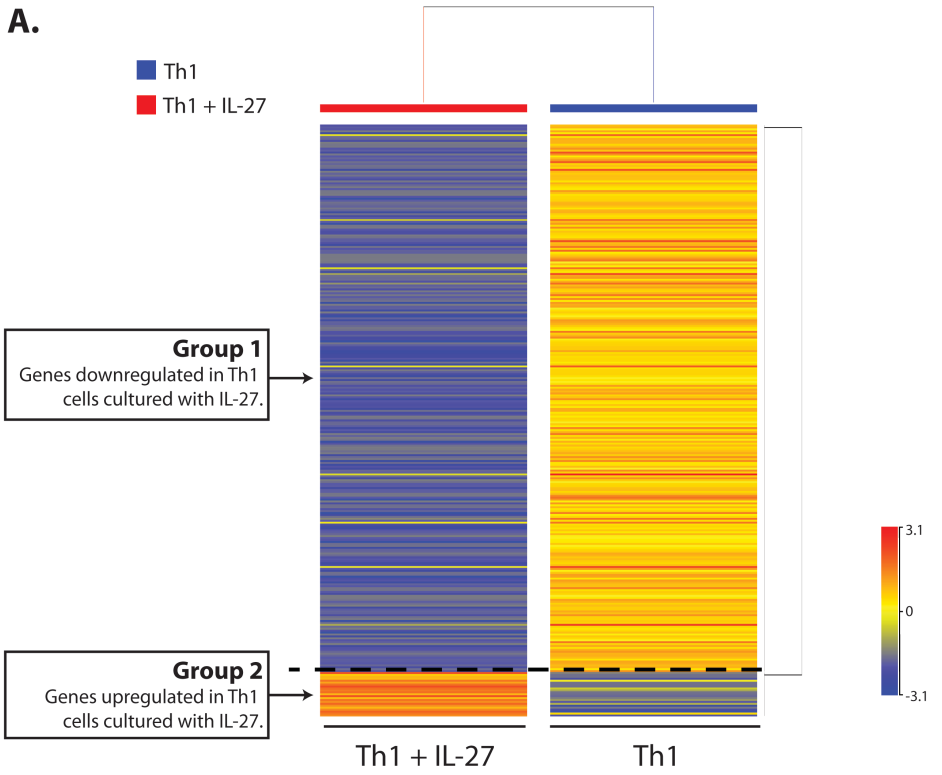


Figure 5A.3 PCA and cluster analysis demonstrates that bulk Th1 and Th1 + IL-27 cells have different gene expression profiles

mRNA extracted and prepared for RNA-Seq as described in the Materials and Methods. **A.** PCA plots of the bulk Th1 and Th1 + IL-27 samples analysed by RNA-Seq. **B.** Unsupervised hierarchical clustering on conditions, of all individual replicates within the bulk Th1 and Th1 + IL-27 subsets. **C.** Unsupervised hierarchical clustering on conditions, of the bulk Th1 and Th1 + IL-27 subsets (repeat data pooled). Unsupervised hierarchical clustering was carried out on the normalised intensity values with a Pearson's centred similarity measure and an Average linkage rule. Colour ranges indicate normalised intensity. The dendrogram represents the relatedness of the samples.



B. Group 1

284 genes downregulated in Th1 cells cultured with IL-27.

Associated GO terms	Associated IPA Pathways	Genes Included
Binding	T cell differentiation, CD28 signalling in Th cells, NFAT signalling	Cyp11a1, Gria3, Cd80, Dgkk, Gzmc, Ifngr2

C. Group 2

23 genes upregulated in Th1 cells cultured with IL-27.

Associated GO terms	Associated IPA Pathways	Genes Included
	Communication between innate & adaptive immune cells, Interferon signalling	Arnt2, Ccl9, Ifit1

Figure 5A.4.1 There are dramatic transcriptional differences between the bulk Th1 and Th1 + IL-27 subsets

mRNA extracted and prepared for RNA-Seq as described in the Materials and Methods. Data from the three biological replicates were pooled. Differentially regulated genes were obtained by taking those that were at least 3-fold up- or downregulated in at least 1 of the 2 samples vs. the baseline (median of all the samples). **A.** This left 307 genes that were subjected to hierarchical clustering on entities and conditions with a Pearson's centred similarity measure and an Average linkage rule. Colour ranges indicate normalised intensity. The dendrogram represents the relatedness of the samples. Genes were separated into 2 groups based on the hierarchical dendrogram. **B.** Group 1 GO terms ($p<0.01$) and IPA pathways ($p<0.01$) with significant overlap, and examples of genes within the group. **C.** Group 2 IPA pathways ($p<0.01$) with significant overlap, and examples of genes within the group. There were no significant GO terms for Group 2.

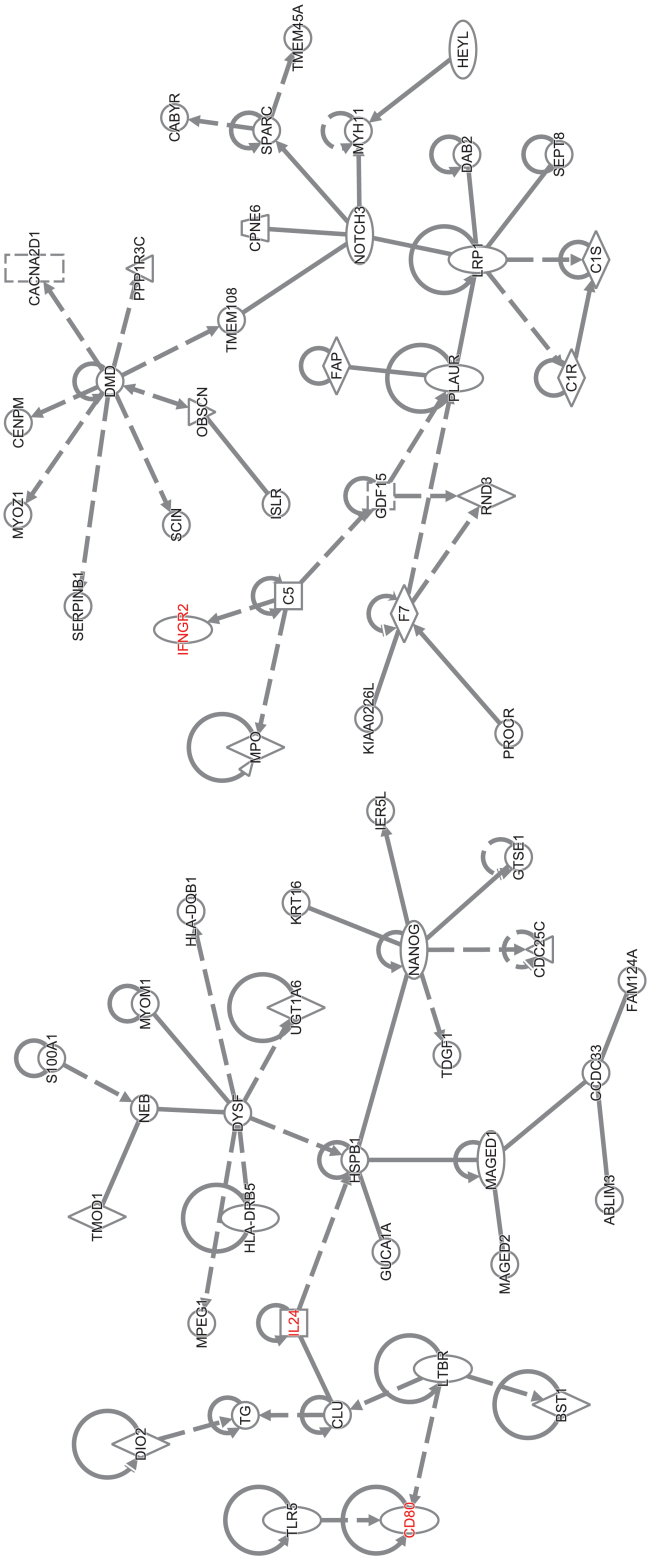


Figure 5A.4.2 Group 1: Network analysis of 284 genes downregulated in Th1 cells cultured with IL-27

IPA network analysis of direct and indirect interactions between genes. Solid lines indicate direct interactions, the dashed lines indicate indirect interactions. Genes in red are those discussed.

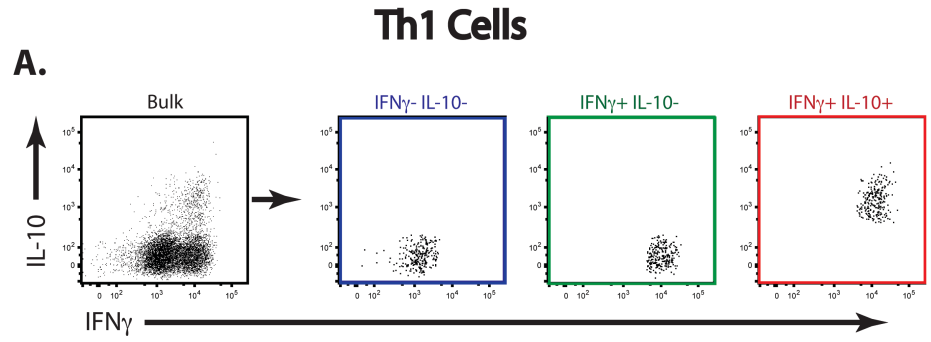
Table 5A.4.1 Group 1: List of 284 genes downregulated in Th1 cells cultured with IL-27

0610040F04Rik	*Cacna2d1*	*Dnahc7a*	*H2-Ab1*
1500009L16Rik	*Calcb*	*Dock7*	*H2-DMb1*
1700003M07Rik	*Camsap3*	*Dpcr1*	*H2-Eb1*
1700007K13Rik	*Cav2*	*Dysf*	*H2-Oa*
2010005H15Rik	*Ccdc120*	*E030011O05Rik*	*Hap1*
2610528A11Rik	*Ccdc159*	*Efr3b*	*Hc*
2900011O08Rik	*Ccdc164*	*F7*	*Hdac11*
4930506M07Rik	*Ccdc33*	*Fah*	*Heyl*
4930550C14Rik	*Cd164l2*	*Fam124a*	*Hhip*
5031414D18Rik	*Cd80*	*Fam13a*	*Hspb1*
5830418P13Rik	*Cdc25c*	*Fam160a1*	*Ier5l*
9830147E19Rik	*Cdh17*	*Fam183b*	*Ifi27l2b*
9830166K06Rik	*Cdhr3*	*Fam84a*	*Ifngr2*
Ablim3	*Cenpm*	*Fap*	*Igfbp7*
Ace	*Chl1*	*Fat4*	*Ikbip*
Adc	*Chrdl2*	*Fbn1*	*Il24*
Adh1	*Ckmt1*	*Fbxo2*	*Iqcg*
Agpat9	*Clec12a*	*Fbxo36*	*Islr*
Ak1	*Clec2l*	*Fhit*	*Islr2*
Akr1b8	*Clu*	*Fzd6*	*Itgb8*
Akr1c12	*Col2a1*	*Gabrr1*	*Kbtbd13*
Akr1e1	*Copz2*	*Gal3st1*	*Klhl23*
Aldh3a1	*Cox6b2*	*Gap43*	*Kmo*
Ankrd29	*Cplx1*	*Gdf15*	*Krt16*
Ankrd33b	*Cpne6*	*Gfpt2*	*Krt17*
Apln	*Cpt1c*	*Gm10406*	*Lama3*
Arhgap23	*Cttnbp2nl*	*Gm11978*	*Lars2*
Arntl2	*Cul7*	*Gm13546*	*Lgals7*
As3mt	*Cx3cr1*	*Gm16548*	*Lima1*
Atp8b3	*Cyp11a1*	*Gm1673*	*Lpin3*
BB031773	*D130043K22Rik*	*Gm4827*	*Lrfn3*
BC018242	*Dab2*	*Gm5111*	*Lrp1*
BC026585	*Dagla*	*Gm5483*	*Lrrc46*
BC055111	*Dapl1*	*Gm8773*	*Lrrc8e*
Bcas1	*Dclk1*	*Gnb3*	*Ltbr*
Bcl2l14	*Dcxr*	*Gprasp2*	*Maged1*
Bfsp2	*Ddc*	*Gpx7*	*Maged2*
Bnip3	*Ddit4l*	*Grhl3*	*Mapk13*
Bst1	*Ddx4*	*Gria3*	*Mcf2l*
C1ql3	*Dgkk*	*Grtp1*	*Mctp1*
C1ra	*Dio2*	*Gtse1*	*Me1*
C1s	*Dkkl1*	*Guca1a*	*Mei4*
Cables1	*Dmd*	*Gucy1a3*	*Mgarp*
Cabyr	*Dnahc10*	*Gzmc*	*Mgmt*

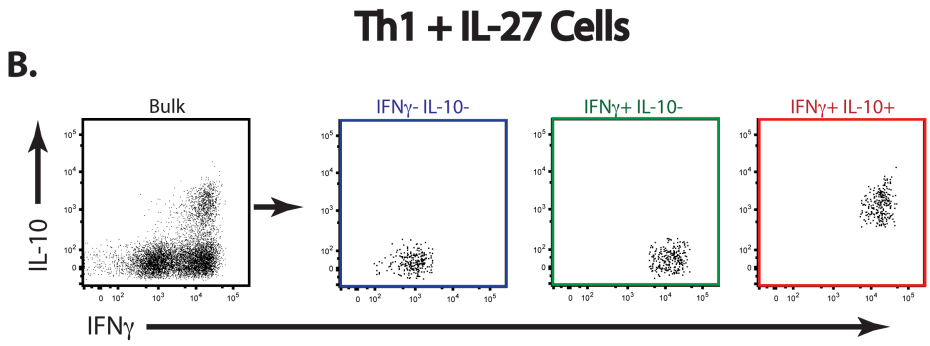
Mir5109	*Pcsk1*	*S100a1*	*Tlr5*
Morn4	*Phldb2*	*Sccpdh*	*Tmem108*
Mpeg1	*Pik3r3*	*Scel*	*Tmem45a*
Mpo	*Plaur*	*Scin*	*Tmod1*
Mx2	*Plch2*	*Scml2*	*Tnfaip6*
Mybl1	*Plekha6*	*Selenbp1*	*Tnfrsf11b*
Mycl1	*Plxna3*	*Selenbp2*	*Tnfsf4*
Myh11	*Podn*	*Sep8*	*Tnp2*
Myh7b	*Pon3*	*Serpinb1a*	*Trf*
Myo16	*Ppp1r3c*	*Shroom3*	*Tro*
Myom1	*Prkar1b*	*Slc17a6*	*Trpm2*
Myoz1	*Prlr*	*Slc30a2*	*Ttc25*
Nags	*Procr*	*Slc7a4*	*Ube2c*
Nanog	*Ptgr1*	*Sparc*	*Ugt1a6a*
Ncrna00086	*Pygl*	*Srms*	*Ugt1a6b*
Neb	*Rad51c*	*Stac2*	*Upk3bl*
Nek2	*Ramp3*	*Stbd1*	*Vill*
Nid1	*Rapsn*	*Styk1*	*Wls*
Notch3	*Rcn3*	*Sv2c*	*Zfp334*
Nov	*Rhod*	*Svip*	*Zfp385a*
Nppc	*Rmrp*	*Syt5*	*Zfp385b*
Ntrk3	*Rnase4*	*Tcea3*	*Zfp449*
Obscn	*Rnd3*	*Tceal8*	*Zfp711*
Ocstamp	*Rnf186*	*Tctex1d1*	*Zfp750*
Osr2	*Robo3*	*Tctn2*	*Zfp768*
P2ry14	*Rpph1*	*Tdgf1*	*Zfp941*
Pafah1b3	*Rsph9*	*Tg*	*Zfyve28*

Table 5A.4.2 Group 2: List of 23 genes upregulated in Th1 cells cultured with IL-27

Arnt2
C030034L19Rik
Ccl9
Cdc42ep1
Cmklr1
Col13a1
Dsg2
E130215H24Rik
Fam101b
Gm14446
Gpr97
Igf2bp1
Klrb1f
Lrrc3b
Mecom
Mmp13
Nrtn
Olfr1033
Rnf32
Slc2a10
Tlr12
Vipr1
Zfp112



Cell Type	Population	Repeat 1	Repeat 2	Repeat 3
Th1	Bulk	100.0	100.0	100.0
Th1	IFNγ- IL-10-	58.8	60.3	66.0
Th1	IFNγ+ IL-10-	30.1	26.8	25.4
Th1	IFNγ+ IL-10+	5.2	4.4	2.0



Cell Type	Population	Repeat 1	Repeat 2	Repeat 3
Th1 + IL-27	Bulk	100.0	100.0	100.0
Th1 + IL-27	IFNγ+ IL-10-	48.7	40.5	44.3
Th1 + IL-27	IFNγ+ IL-10-	36.3	40.3	23.6
Th1 + IL-27	IFNγ+ IL-10+	10.9	9.7	13.5

Figure 5B.1 Subpopulations of Th1 and Th1 + IL-27 subsets

C57BL/6 naive CD4+CD62L+CD44loCD25- cells were driven with plate-bound anti-CD3 and soluble anti-CD28. Th1 cells were polarised with IL-12 and anti-IL-4 in the presence or absence of IL-27, and were assessed after 7 days of polarization *in vitro*. Cells were restimulated for ICS as described in the Materials and Methods. Plots of sorted populations by flow cytometric analysis of intracellular cytokine staining. Tables showing the percentage of the total number of cells that each cytokine producing subpopulation represented for each of the three repeat experiments. The bulk is 100% of all CD4+ live cells as described in the Materials and Methods. **A.** Subpopulations within the Th1 subset. **B.** Subpopulations within the Th1 + IL-27 subset.

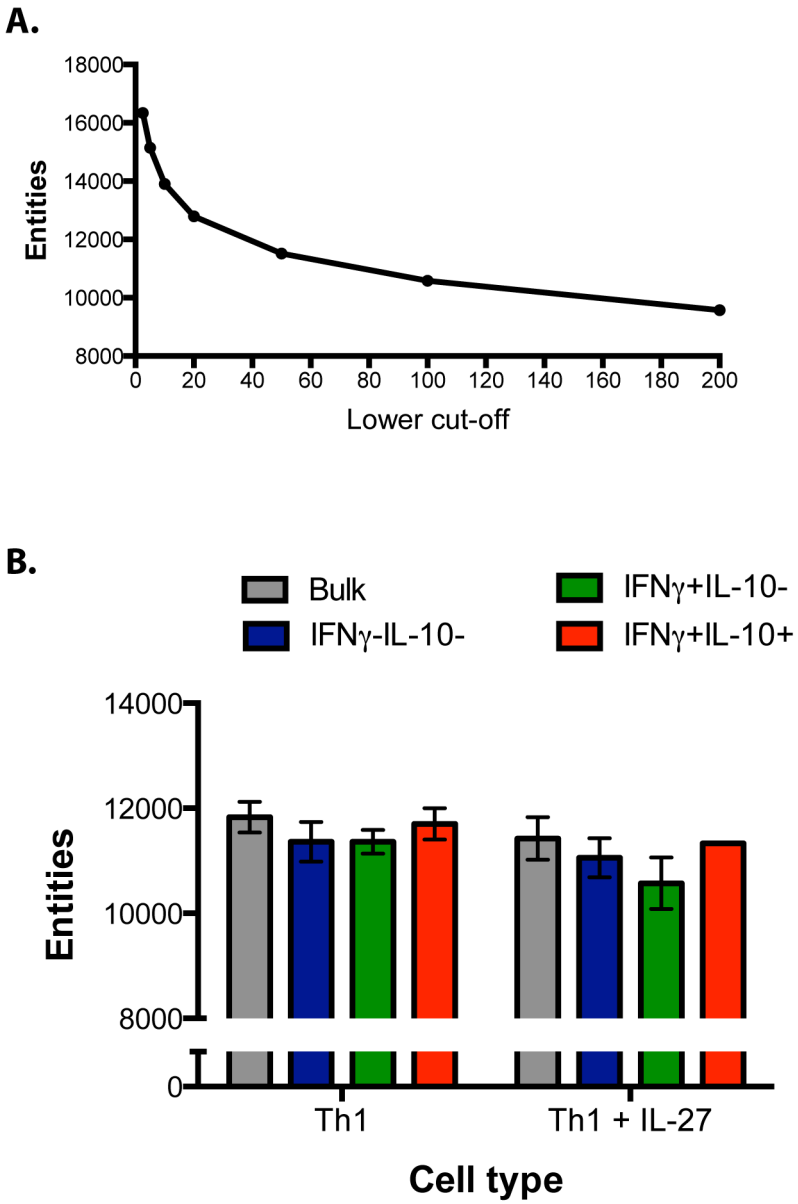


Figure 5B.2 Determining the optimal lower cut-off of reads for expression threshold of Th1 and Th1 + IL-27 RNA-Seq samples

Cell populations are described in Figure 5B.1, mRNA extracted and prepared for RNA-Seq as described in Materials and Methods. Using Strand NGS software reads were aligned to the transcriptome & Genome (mm10, RefSeq annotation, 95% identity, max 5% gaps, 1 read only if duplicate) and normalisation with DeSeq and no Baseline. Upper cut-off 1165880.5 (where at least 1 out of 24 samples have values within cut-off). **A.** The number of entities passing expression thresholds with indicated lower cut-offs. All the Th1 and Th1 + IL-27 samples were pooled for this test. **B.** Using an expression threshold with a lower cut-off of 20 reads, the number of entities in each repeat of each subpopulation within the Th1 and Th1 + IL-27 subsets was determined.

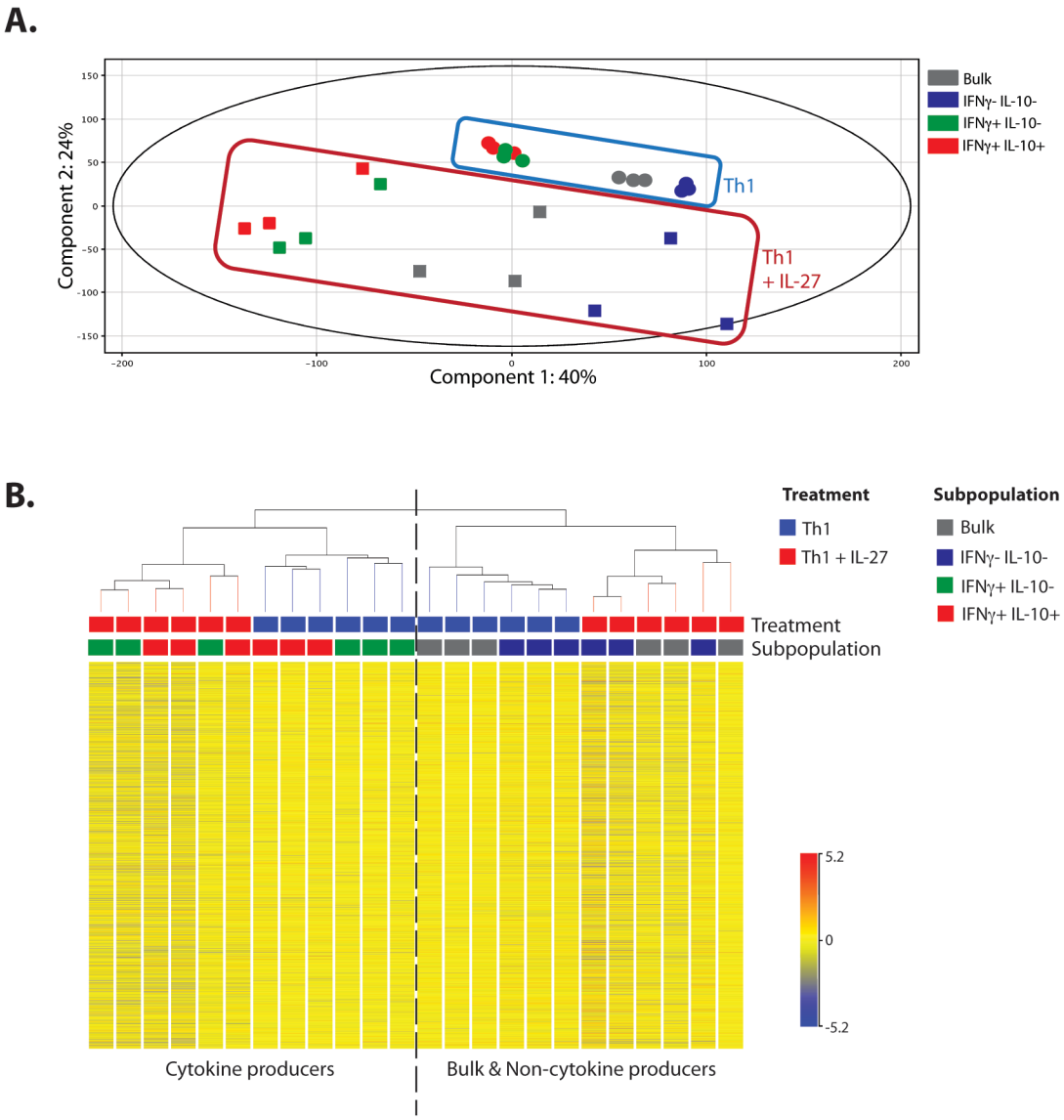


Figure 5B.3 PCA and cluster analysis demonstrates that Th1 and Th1 + IL-27 cells have different gene expression profiles

Cell populations are described in Figure 5B.1, mRNA extracted and prepared for RNA-Seq as described in the Materials and Methods. **A.** PCA plots of different intracellular cytokine producing subpopulations within the Th1 and Th1 + IL-27 samples analysed by RNA-Seq. **B.** Unsupervised hierarchical clustering on conditions, of all individual replicates within the Th1 and Th1 + IL-27 subsets, was carried out on the normalised intensity values with a Pearson's centred similarity measure and an Average linkage rule. Colour ranges indicate normalised intensity. The dendrogram represents the relatedness of the samples.

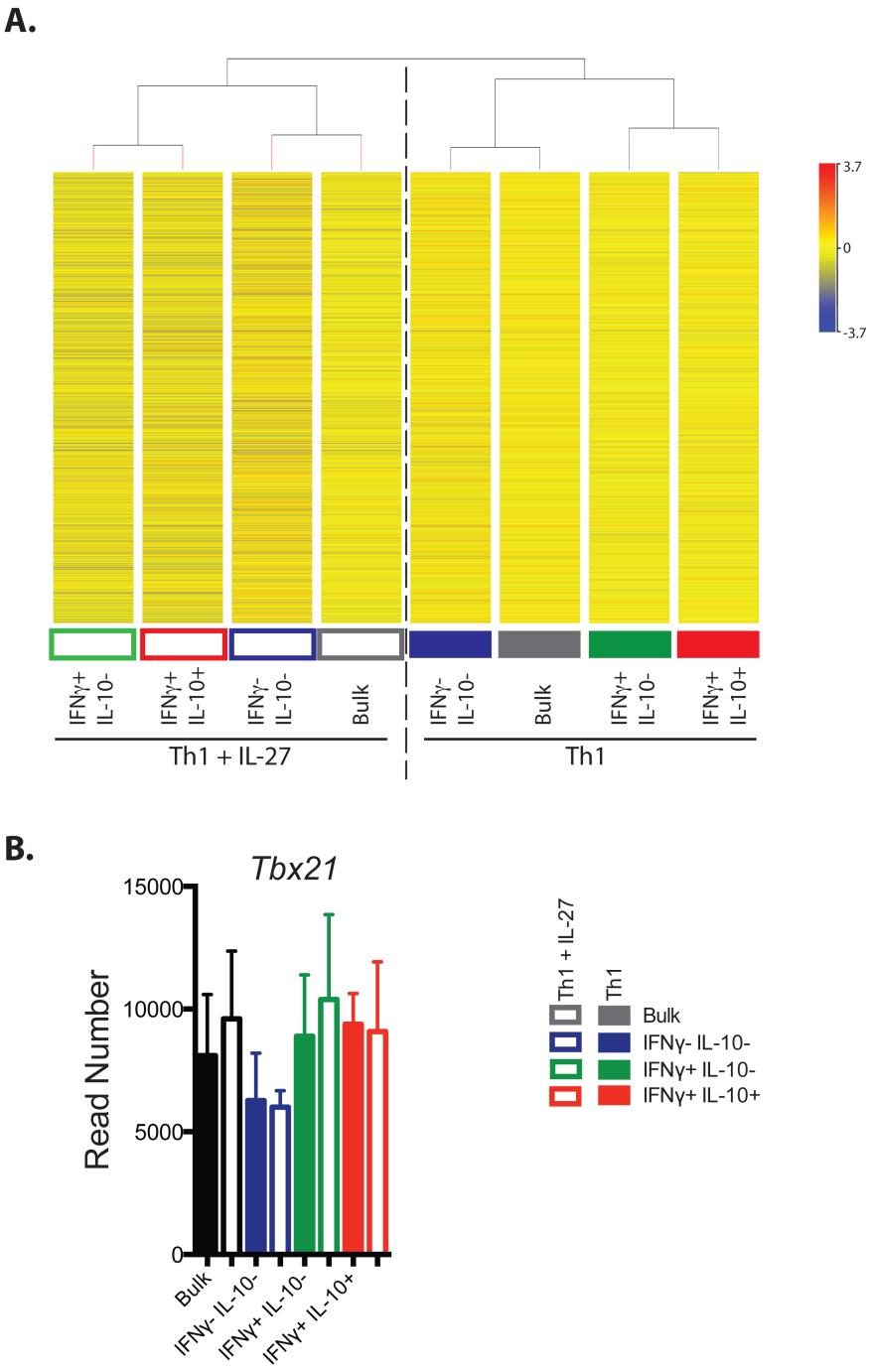


Figure 5B.4 Clustering analysis demonstrates that Th1 cells and Th1 + IL-27 cells are distinct and have transcriptional differences

Cell populations are described in Figure 5B.1, mRNA extracted and prepared for RNA-Seq as described in the Materials and Methods. Data from the three biological replicates were pooled. **A.** Unsupervised hierarchical clustering on conditions, of each subpopulations within the Th1 and Th1 + IL-27 subsets, was carried out on the normalised intensity values with a Pearson's centred similarity measure and an Average linkage rule. Colour ranges indicate normalised intensity. The dendrogram represents the relatedness of the samples. **B.** The read number of *Tbx21* in each subpopulation.

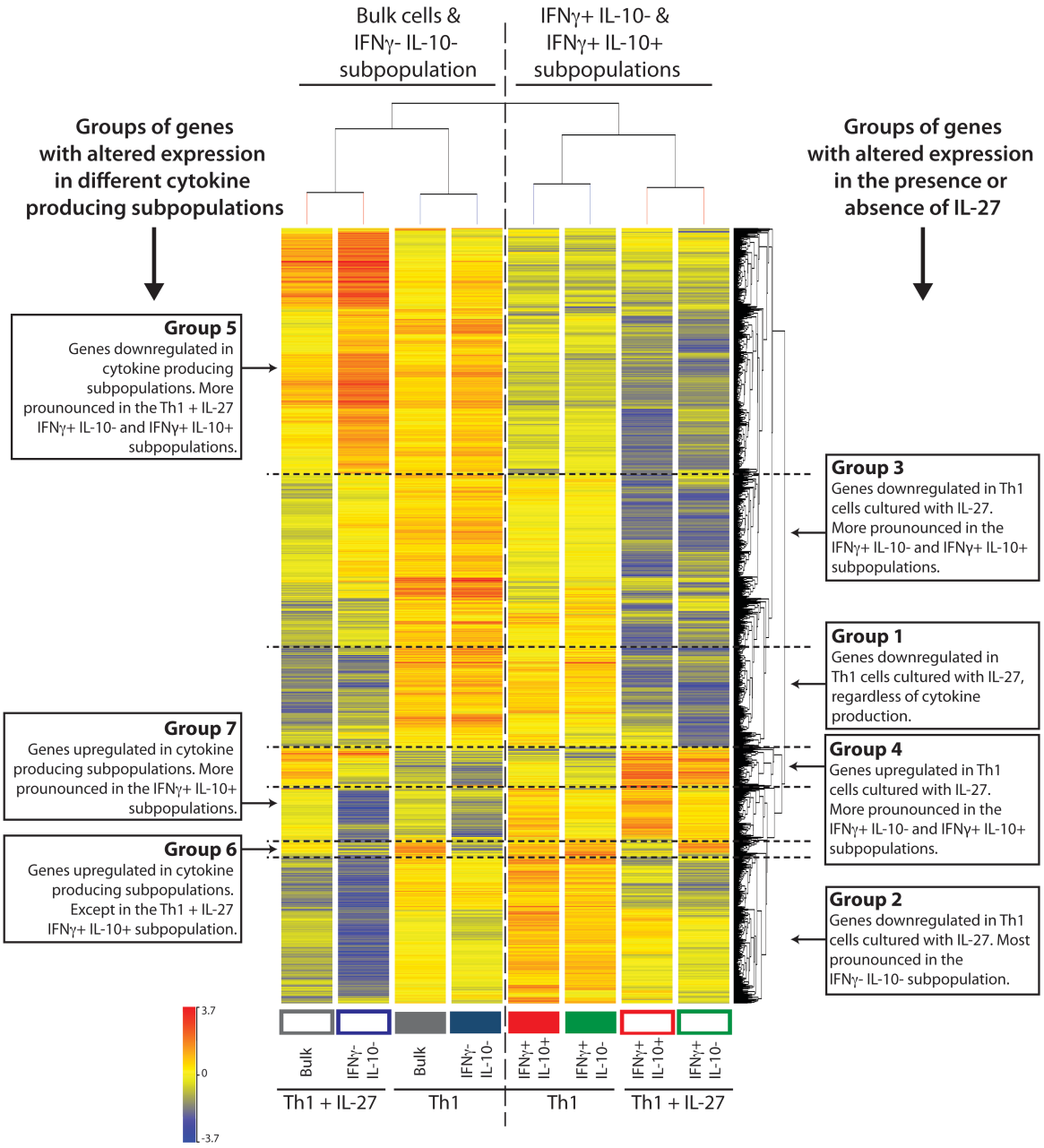
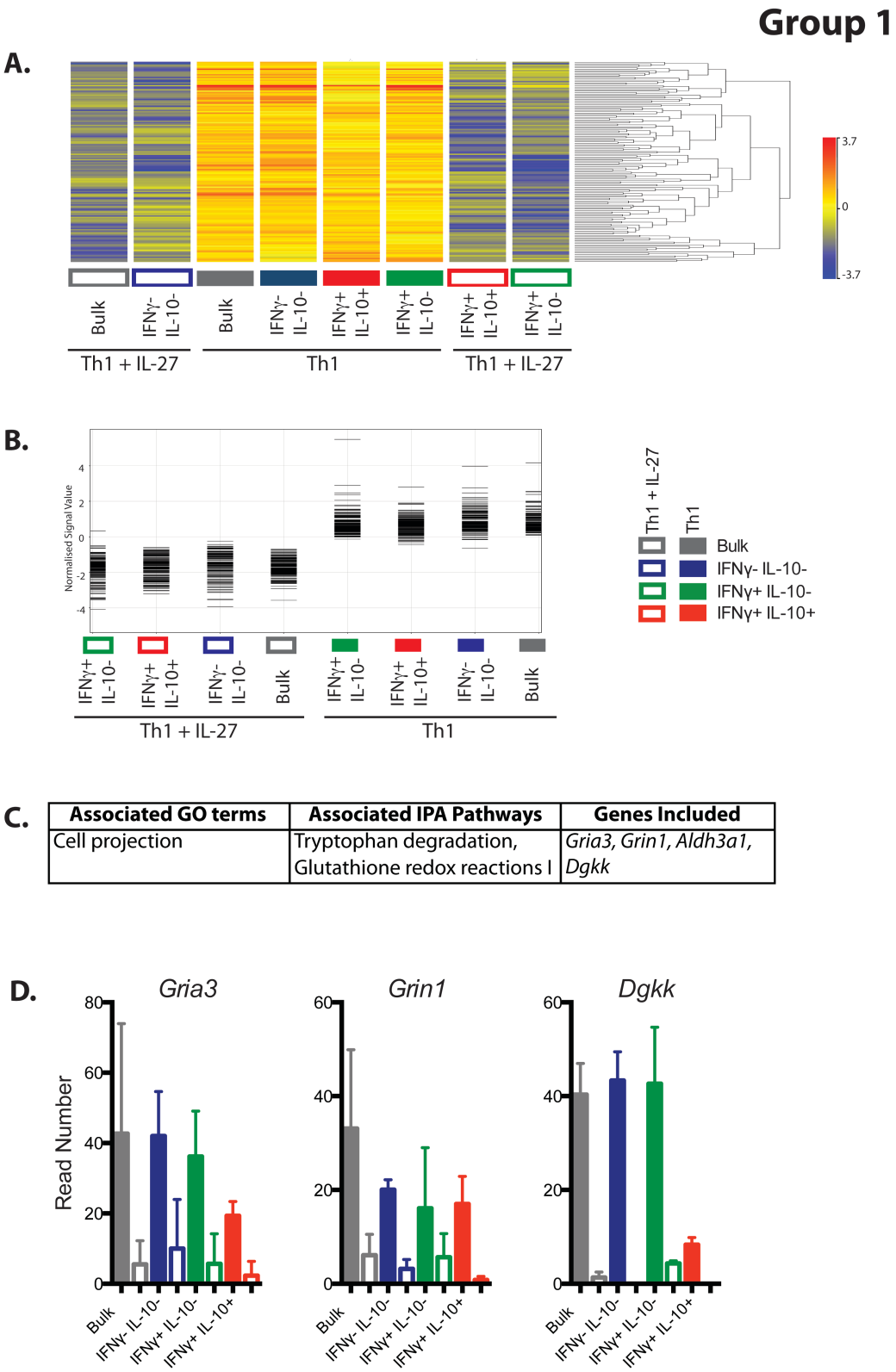


Figure 5B.5 There are dramatic transcriptional differences between the different intracellular cytokine producing subpopulations within the Th1 and Th1 + IL-27 subsets

Cell populations are described in Figure 5B.1, mRNA extracted and prepared for RNA-Seq as described in the Materials and Methods. Data from the three biological replicates were pooled. Differentially regulated genes were obtained by taking those that were at least 3-fold up- or downregulated in at least 1 of the 8 samples vs. the baseline (median of all the samples). This left 1944 genes that were subjected to hierarchical clustering on entities and conditions with a Pearson's centred similarity measure and an Average linkage rule. Colour ranges indicate normalised intensity. The dendrogram represents the relatedness of the samples. Genes were separated into 7 groups based on the hierarchical dendrogram and experimental hypothesis.



C. Associated GO terms	Associated IPA Pathways	Genes Included
Cell projection	Tryptophan degradation, Glutathione redox reactions I	*Gria3, Grin1, Aldh3a1, Dgkk*

Figure 5B.6.1 Group 1: 146 genes downregulated in Th1 cells cultured with IL-27, regardless of cytokine production

A. Genes in Group 1 from hierarchical clustering in Figure 5B.5. **B.** Expression profile of group. **C.** GO terms ($p<0.05$) and IPA pathways ($p<0.01$) with significant overlap, and examples of genes within the group. **D.** The read number of selected genes in each subpopulation.

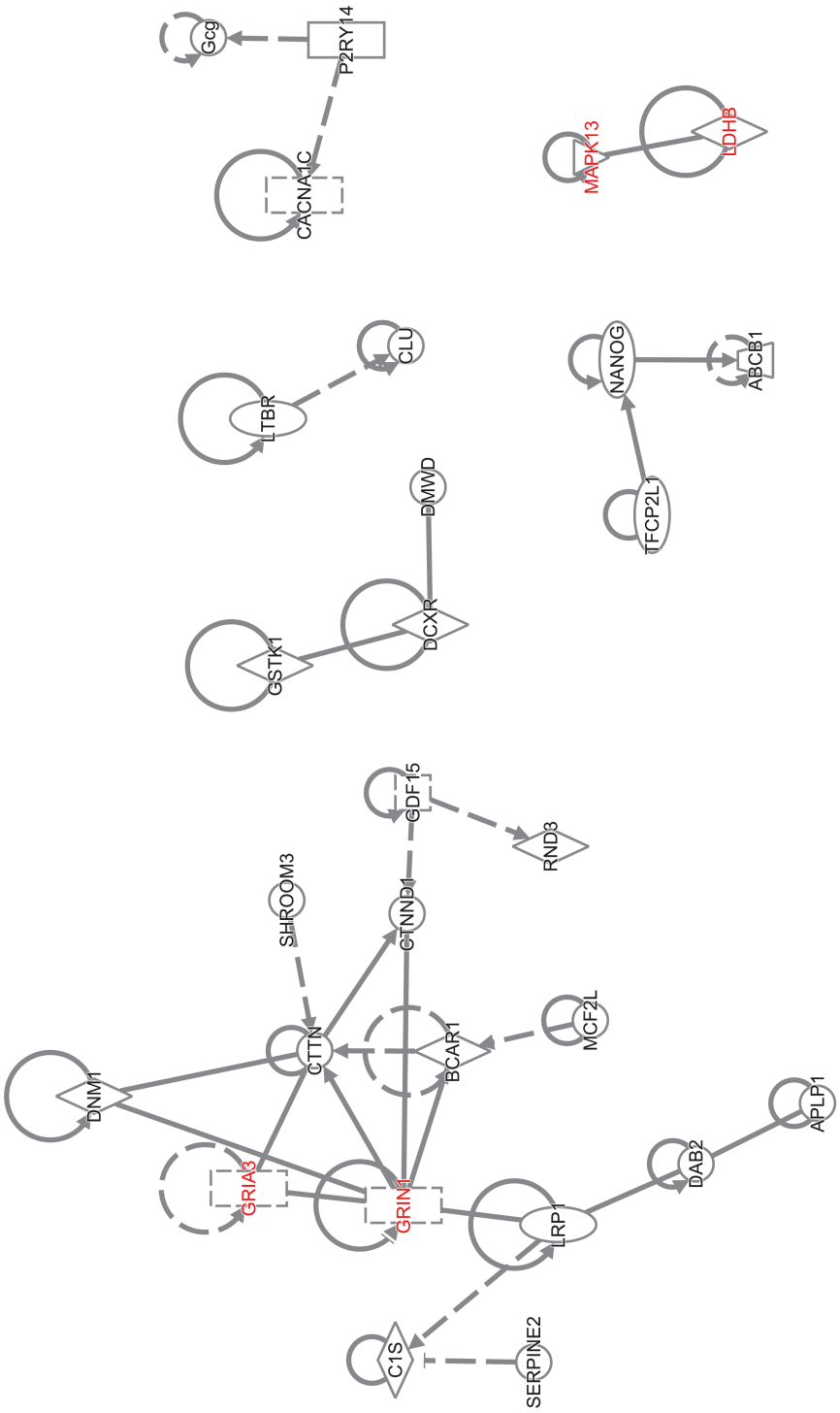


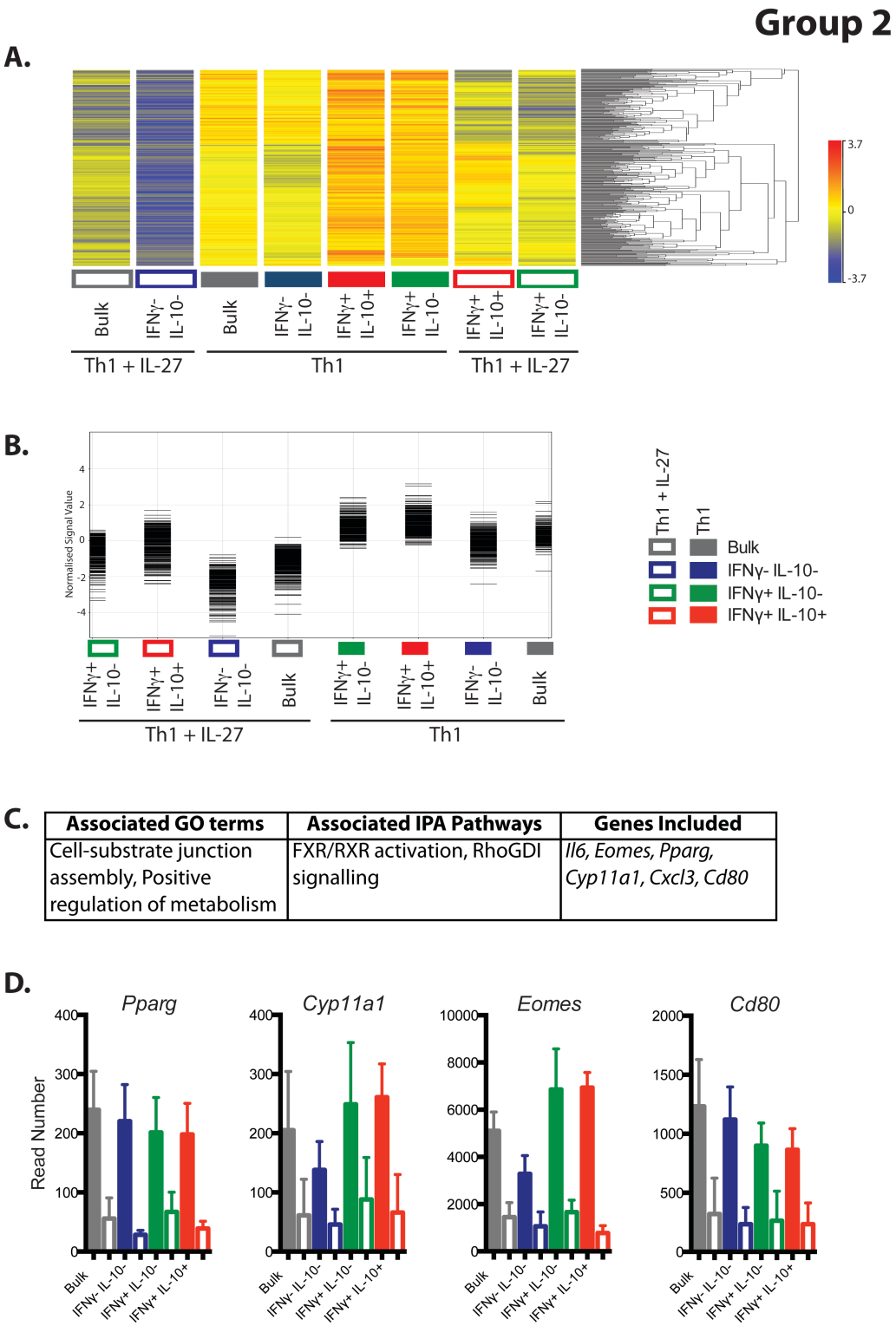
Figure 5B.6.2 Group 1: Network analysis of 146 genes downregulated in Th1 cells cultured with IL-27, regardless of cytokine production

IPA network analysis of direct and indirect interactions between genes. Solid lines indicate direct interactions, the dashed lines indicate indirect interactions. Genes in red are those discussed.

Table 5B.6.1 Group 1: List of 146 genes downregulated in Th1 cells cultured with IL-27, regardless of cytokine production

Genes highlighted in blue are those also found in Group 1 of Table 5A.4.1.

1500009L16Rik	*Dcxr*	*Itgb8*	*Rnf186*
2010005H15Rik	*Ddc*	*Kmo*	*Rsph9*
4930500J02Rik	*Ddit4l*	*Ldhb*	*S100a1*
5031414D18Rik	*Dgkk*	*Leprel2*	*Sccpdh*
Abcb1a	*Dmd*	*Liph*	*Scml2*
Ablim3	*Dmwd*	*Lpin3*	*Scn9a*
Ace	*Dnahc10*	*Lrp1*	*Selenbp1*
Akr1c12	*Dnahc7a*	*Ltbr*	*Selenbp2*
Aldh3a1	*Dnm1*	*Maged1*	*Selp*
Aldoc	*Efr3b*	*Mapk13*	*Serpinb6a*
Ankrd29	*Ehd2*	*Mcf2l*	*Serpine2*
Apln	*Enkur*	*Megf8*	*Shroom3*
Aplp1	*F5*	*Mgmt*	*Sspo*
Arhgap23	*Fah*	*Morn2*	*Susd1*
Avpi1	*Faim3*	*Morn4*	*Syngr4*
BB031773	*Fam183b*	*Mx1*	*Syt5*
Bbs5	*Fam20c*	*Mycl1*	*Tal2*
Bcar1	*Fam84a*	*Myh7b*	*Tcea3*
C1ql3	*Fbn1*	*Nags*	*Tctex1d1*
C1s	*Fbxo36*	*Nanog*	*Tctn2*
Cables1	*Fhit*	*Ncrna00086*	*Tfcp2l1*
Cabyr	*Gcg*	*Neb*	*Tlr5*
Cacna1c	*Gdf15*	*Nov*	*Tmem108*
Ccdc164	*Gm10406*	*Ntn3*	*Tmem35*
Ccdc40	*Gm16548*	*Oas1a*	*Tnp2*
Ccr4	*Gm17384*	*Oas1g*	*Trf*
Ccr8	*Gm19619*	*P2ry14*	*Tro*
Cd164l2	*Gm5483*	*Pdxk-ps*	*Trpm2*
Cdh15	*Gm8773*	*Plch2*	*Ttc25*
Ckmt1	*Gpr162*	*Pon3*	*Upk3bl*
Clec2l	*Gpx7*	*Prkar1b*	*Vill*
Clu	*Gria3*	*Procr*	*Vipr2*
Copz2	*Grin1*	*Ptgr1*	*Zfp365*
Cox6b2	*Gstk1*	*Rabl2*	*Zfp870*
Ctnnd1	*Guca1a*	*Rapsn*	*Zfp941*
Cttn	*H2-Ab1*	*Rcn3*	
Dab2	*H2-Oa*	*Rnd3*	



Associated GO terms	Associated IPA Pathways	Genes Included
Cell-substrate junction assembly, Positive regulation of metabolism	FXR/RXR activation, RhoGDI signalling	*Il6, Eomes, Pparg, Cyp11a1, Cxcl3, Cd80*

Figure 5B.7.1 Group 2: 349 genes downregulated in Th1 cells cultured with IL-27. More pronounced in Th1 + IL-27 IFNγ- IL-10- subpopulation

A. Genes in Group 2 from hierarchical clustering in Figure 5B.5. **B.** Expression profile of group. **C.** GO terms ($p<0.05$) and IPA pathways ($p<0.01$) with significant overlap, and examples of genes within the group. **D.** The read number of selected genes in each subpopulation.

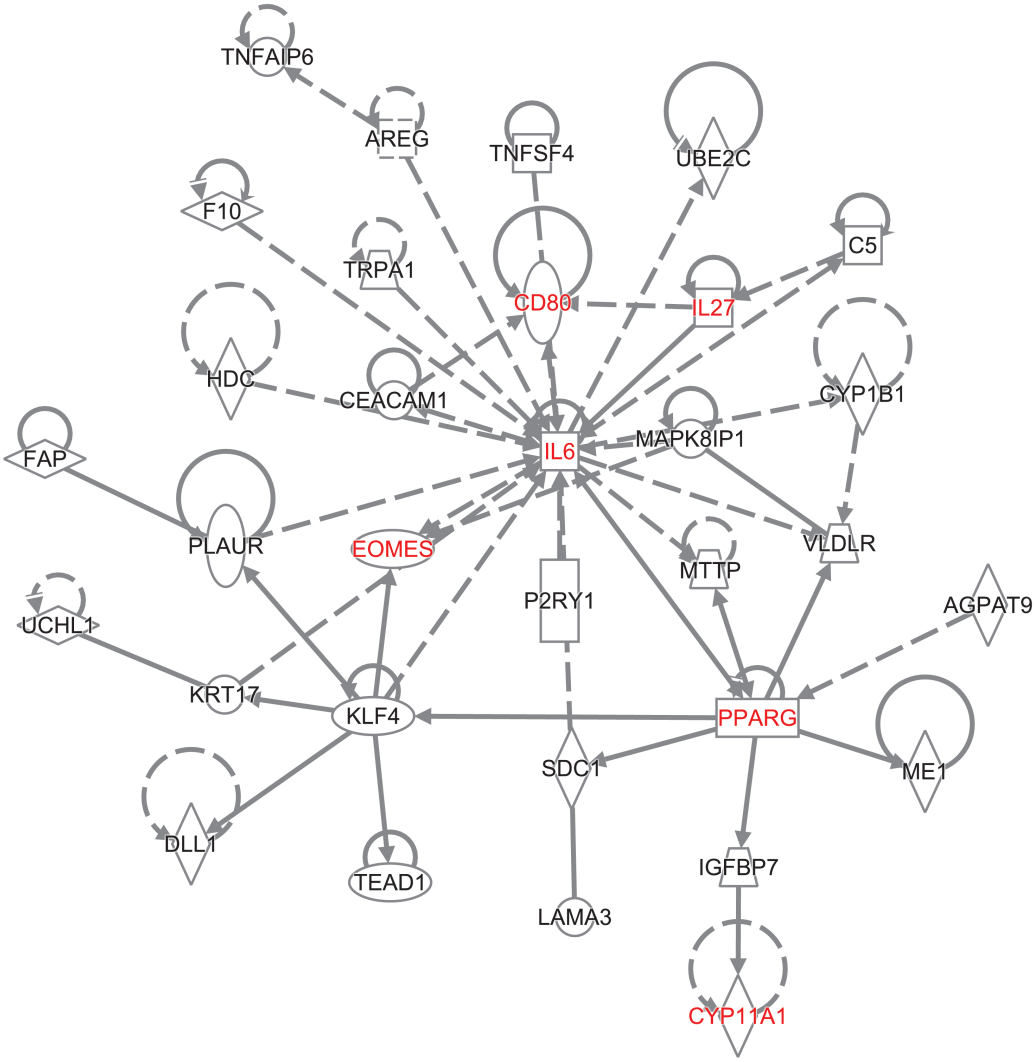


Figure 5B.7.2 Group 2: Network analysis of 349 genes downregulated in Th1 cells cultured with IL-27. More pronounced in Th1 + IL-27 IFNγ- IL-10- subpopulation

IPA network analysis of direct and indirect interactions between genes. Solid lines indicate direct interactions, the dashed lines indicate indirect interactions. Genes in red are those discussed.

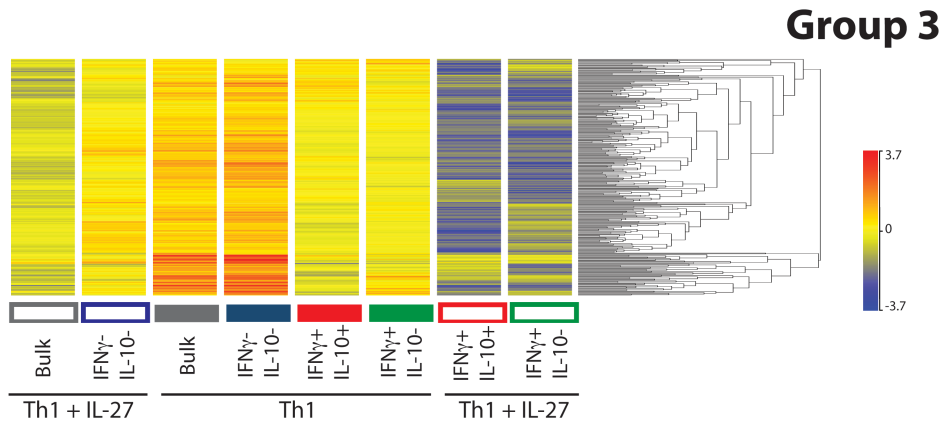
Table 5B.7.1 Group 2: List of 349 genes downregulated in Th1 cells cultured with IL-27. More pronounced in Th1 + IL-27 IFNγ- IL-10- subpopulation

Genes highlighted in blue are those also found in Group 1 of Table 5A.4.1.

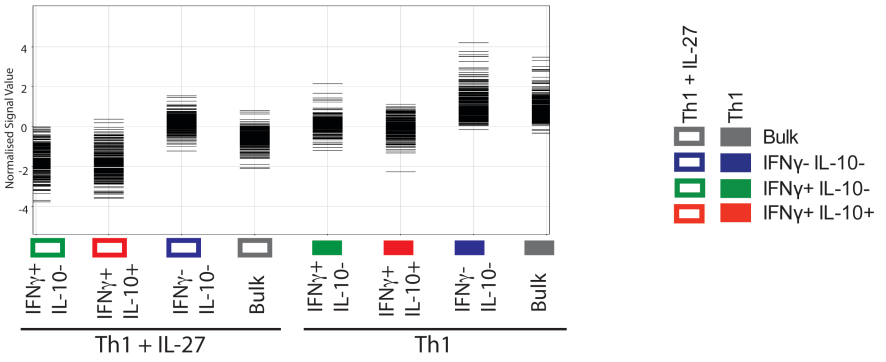
1700016G22Rik	*Cacna2d1*	*E030011O05Rik*	*Gm13889*
1700101I11Rik	*Calcb*	*E430016F16Rik*	*Gm14047*
2810408I11Rik	*Camkv*	*Eda2r*	*Gm15663*
3110015C05Rik	*Car8*	*Edaradd*	*Gm16596*
4930506M07Rik	*Ccr1*	*Emp2*	*Gm17745*
4933402N22Rik	*Cd109*	*Enpep*	*Gm19510*
5830418P13Rik	*Cd63*	*Eomes*	*Gm2663*
9830001H06Rik	*Cd80*	*Ermn*	*Gm2897*
Abcb4	*Cd83*	*Esrp2*	*Gm3558*
Ablim2	*Cdc20*	*Exo1*	*Gm3696*
Acsl6	*Cdcp1*	*Exph5*	*Gm5077*
Actbl2	*Cdh17*	*F10*	*Gm5475*
Adam30	*Ceacam1*	*F7*	*Gm6455*
Adamts3	*Ch25h*	*Fads3*	*Gm6460*
Adssl1	*Chn1*	*Fam124b*	*Gm8267*
Agpat9	*Col16a1*	*Fam43a*	*Gm867*
AI464131	*Col27a1*	*Fam71f2*	*Gna14*
AI836003	*Cpne6*	*Fap*	*Gnb3*
Ak1	*Crabp2*	*Fat4*	*Gpr3*
Akr1b8	*Csf2rb2*	*Fcrl1*	*Grb10*
Akr1c18	*Cx3cl1*	*Fermt2*	*Gspt2*
Amica1	*Cxcl2*	*Fhdc1*	*Gstm5*
Amotl2	*Cxcl3*	*Fhl2*	*Gzmb*
Angptl2	*Cyp11a1*	*Fkbp9*	*Gzmc*
Ankrd33b	*Cyp1b1*	*Flt1*	*Havcr2*
Ankrd34b	*Cyr61*	*Flywch2*	*Hc*
Arc	*D10Bwg1379e*	*Fmnl2*	*Hdc*
Areg	*Dab2ip*	*Fn1*	*Heyl*
Arhgap6	*Dagla*	*Foxc1*	*Hhip*
Arhgef9	*Dclk1*	*Foxf1*	*Hhipl2*
Atcay	*Ddr2*	*Fsd1l*	*Hmx3*
Atoh8	*Depdc7*	*Fut4*	*Hormad2*
Atp1b2	*Dhrs9*	*Fzd6*	*Ifih1*
Atp8b4	*Diap3*	*Gabrr1*	*Ifitm2*
Basp1	*Dixdc1*	*Galnt14*	*Igf2*
BC055111	*Dlc1*	*Gfpt2*	*Igfbp7*
Bst1	*Dleu7*	*Gjb2*	*Igsf9b*
C2	*Dll1*	*Gldc*	*Il13*
C2cd4b	*Dll3*	*Glrp1*	*Il1r1*
C3	*Dnase1l3*	*Gls2*	*Il1rn*
C430002N11Rik	*Dsp*	*Gm10409*	*Il20ra*
Cabp1	*Dtx1*	*Gm11978*	*Il24*
Cacna1s	*Dusp18*	*Gm12709*	*Il27*

Il31
Il33
Il6
Inhba
Iqcf3
Isg15
Islr
Jsrp1
Kcnh3
Kcnj12
Kctd15
Kif15
Klf4
Klhl23
Klre1
Klrk1
Krt16
Krt17
Lama3
Lctl
Lgals7
Lhfpl2
Lingo1
Lipg
Ltbp1
Ltbp3
Mafb
Map3k6
Mapk12
Mapk8ip1
Mdga1
Me1
Mmp10
Mnda
Mtap7d3
Mttp
Myh7
Mylk
Myo15
Myo1e
Ncrna00085
Neo1
Nid1
Nos2
Nppc
Nrg1
Nrn1
Nrp1
Nts
Nupr1
Oaf
Ocstamp
Olfr266
Omp
Osbpl3
Osr2
Otx1
Ovol2
P2rx5
P2ry1
P2ry12
P4ha2
Palld
Pcdh1
Pcdh7
Pdgfa
Pdgfc
Pdzk1ip1
Penk
Perp
Pla1a
Plagl1
Plaur
Plcl1
Plek2
Plk2
Pln
Pparg
Ppfibp2
Ppp2r3a
Prkcdbp
Prss2
Ptgs1
Ptgs2
Ptprk
Ptprn
Ptrf
Rab13
Ramp3
Rasgef1b
Rgs2
Rgs8
Rgs9
Rimbp3
Ripk4
Rnf217
Rsc1a1
S100a2
S100a3
S1pr3
Scel
Scg2
Scin
Sdc1
Sema4c
Sema6d
Sep03
Serpinb1b
Serpinb6b
Serpinb6c
Serpinb9
Serpinb9b
Sgip1
Sgms2
Siglecg
Slc13a3
Slc15a3
Slc17a6
Slc1a2
Slc4a4
Slc5a5
Slc6a17
Slc7a11
Slc7a3
Soat2
Sort1
Sp7
Speer1-ps1
Spem1
Spink2
Spint1
Spns2
Spry2
Sstr3
Stbd1
Stc1
Stra6
Stx1b
Sulf2
Sv2c
Tal1
Tead1
Tgfb2
Timp1
Tjp2
Tm4sf19
Tmem132a
Tmem252
Tmtc2
Tnfaip2
Tnfaip6
Tnfsf11
Tnfsf4
Tnnc2
Tnnt2
Trhr2
Trim16
Trim71
Trip13
Trpa1
Tslp
Ttc39c
Txlnb
Ube2c
Uchl1
Upb1
Vash2
Vldlr
Vwa1
Wdr63
Wisp1
Xirp1
Ybx2
Zc3h12c
Zdbf2
Zfp105
Zfp334

A.



B.



C.

Associated GO terms	Associated IPA Pathways	Genes Included
	NFAT signalling, Aryl hydrocarbon receptor signalling, Tryptophan degradation	*Aldh1l1, Aldh5a1, Ido1, Ar, Ifngr2*

D.

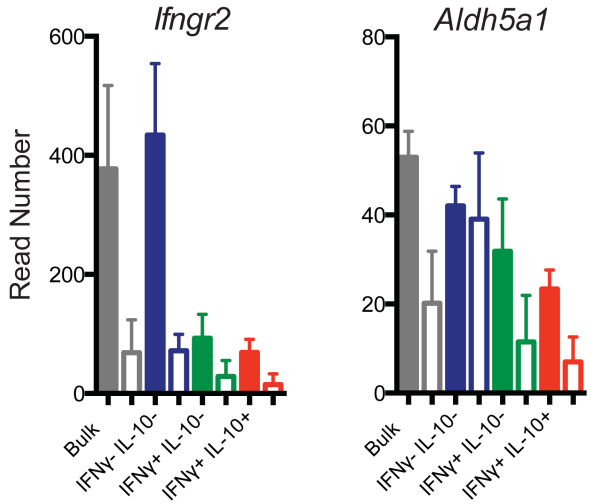


Figure 5B.8.1 Group 3: 299 genes downregulated in Th1 cells cultured with IL-27. More pronounced in Th1 + IL-27 cytokine producing subpopulations

A. Genes in Group 3 from hierarchical clustering in Figure 5B.5. **B.** Expression profile of group. **C.** IPA pathways ($p<0.01$) with significant overlap, and examples of genes within the group (no significant GO terms). **D.** The read number of selected genes in each subpopulation.

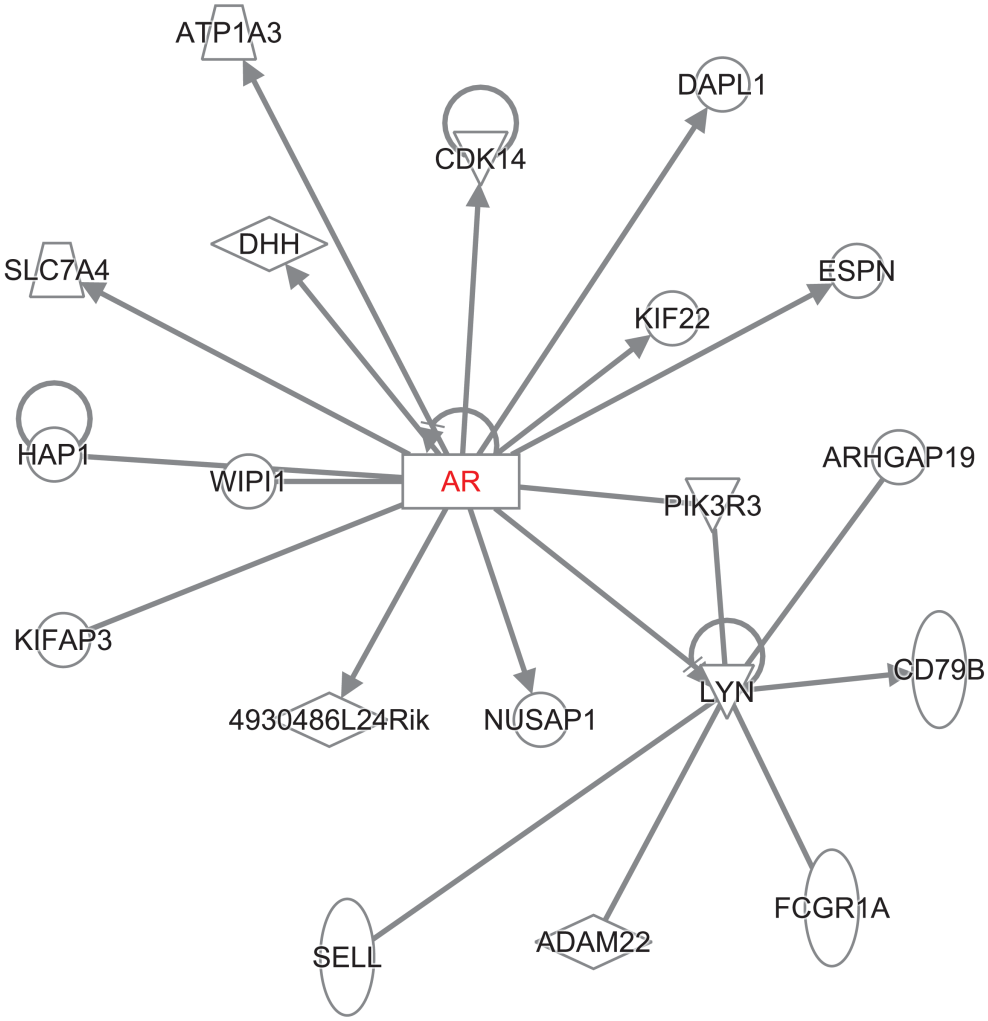


Figure 5B.8.2 Group 3: Network analysis of 299 genes downregulated in Th1 cells cultured with IL-27. More pronounced in Th1 + IL-27 cytokine producing subpopulations

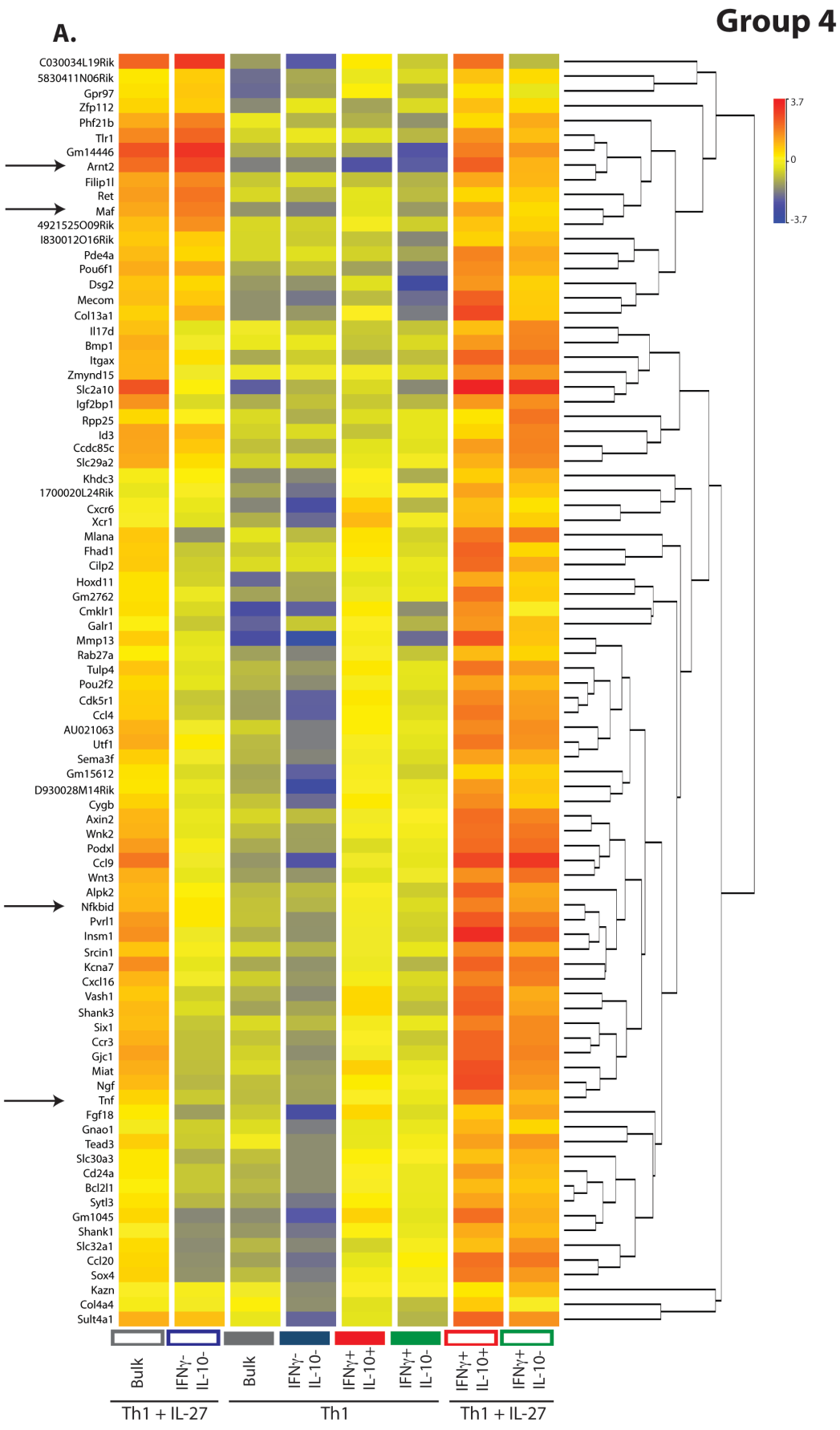
IPA network analysis of direct interactions between genes. Genes in red are those discussed.

Table 5B.8.1 Group 3: List of 299 genes downregulated in Th1 cells cultured with IL-27. More pronounced in Th1 + IL-27 cytokine producing subpopulations

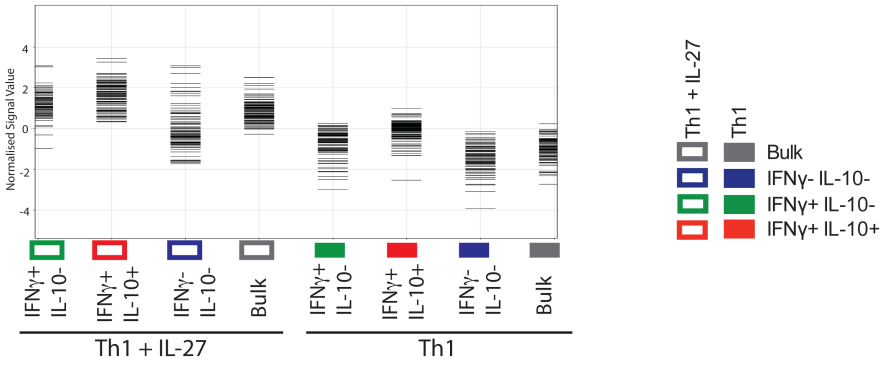
Genes highlighted in blue are those also found in Group 1 of Table 5A.4.1.

1110019D14Rik	*Atp6v0a1*	*Dgat1*	*Gpr180*
1110032A03Rik	*Bace2*	*Dgcr6*	*Gpr34*
1700021K19Rik	*Bbs9*	*Dhh*	*Gpr55*
1700066B19Rik	*BC035044*	*Dhrs1*	*Gprasp2*
2210404O09Rik	*Bicd1*	*Dlg3*	*Gpx4*
2210408F21Rik	*Bivm*	*Dnajc18*	*Grhl3*
2210408I21Rik	*Bnip3*	*Dnm3*	*Gstm4*
2410004P03Rik	*Bnipl*	*Dusp19*	*Gucy2e*
2610019F03Rik	*C1qtnf1*	*Dync2li1*	*Gyg*
2610528A11Rik	*C1qtnf4*	*Ebpl*	*H2-DMb1*
2810029C07Rik	*C8g*	*Ech1*	*H2-DMb2*
2810403D21Rik	*Cacna2d2*	*Eci2*	*H2afv*
2810408A11Rik	*Cby1*	*Eci3*	*Haao*
2900011O08Rik	*Ccdc11*	*Ect2l*	*Hap1*
4930486L24Rik	*Ccdc34*	*Efcab11*	*Hdac11*
4930539E08Rik	*Cchcr1*	*Egf*	*Hexb*
4930579G24Rik	*Cd200r1*	*Ehbp1*	*Hist1h2bc*
4931414P19Rik	*Cd226*	*Eif2ak2*	*Hpse*
6330403K07Rik	*Cd79b*	*Enpp1*	*Hpx*
6430548M08Rik	*Cdca3*	*Ephx4*	*Hspb1*
AB124611	*Cdk14*	*Ercc5*	*Hspg2*
Abcc3	*Cdkl4*	*Erp27*	*Ica1*
Abcg4	*Cdr2l*	*Espn*	*Ido1*
Abhd4	*Celf5*	*Evi5l*	*Ifi27l2b*
Acacb	*Cep19*	*Exd1*	*Ifi44*
Acvr2b	*Chrdl2*	*Extl1*	*Ifngr2*
Adam22	*Cldn10*	*Fam111a*	*Ift140*
Adamtsl5	*Clec12a*	*Fam124a*	*Ift81*
Adc	*Clip3*	*Fam13a*	*Ikbip*
Adh1	*Cntln*	*Fam160a2*	*Ikzf2*
Agl	*Cul7*	*Fam161b*	*Immp1l*
Aipl1	*Cxcl11*	*Fam64a*	*Insl5*
Akr1b10	*Cybrd1*	*Fcgr1*	*Iqcd*
Aldh1l1	*Cyp27a1*	*Fech*	*Irf6*
Aldh5a1	*Cyp4f16*	*Galr2*	*Itgb3bp*
Ampd3	*D130043K22Rik*	*Gm16197*	*Itih5*
Ankmy2	*D3Ertd254e*	*Gm5595*	*Ivd*
Aox3l1	*Dapl1*	*Gm766*	*Kctd7*
Ar	*Dbn1*	*Gna15*	*Khk*
Arhgap19	*Dbp*	*Gnal*	*Kif18a*
Asah2	*Dcun1d3*	*Gnaz*	*Kif22*
Asb1	*Decr1*	*Gpr152*	*Kifap3*
Atp1a3	*Depdc1b*	*Gpr179*	*Lancl1*

Ldb3	*Oas2*	*Rab33a*	*Thnsl2*
Leprel4	*Oip5*	*Rab4a*	*Thsd1*
Lhpp	*Olfr60*	*Rap1gap*	*Tle2*
Lrrc28	*Olfr920*	*Rbm20*	*Tmem106c*
Lrrc48	*Ophn1*	*Rec8*	*Tmem52*
Lyn	*Osgepl1*	*Rhod*	*Tmsb15l*
Lynx1	*P4htm*	*Rin2*	*Tmtc1*
Macrod2	*Paqr3*	*Rnase4*	*Tox*
Mansc1	*Parp12*	*Rnf135*	*Tpmt*
Mcee	*Pcx*	*Rsu1*	*Trim6*
Mctp1	*Pde5a*	*Rtkn2*	*Trpv6*
Mef2b	*Pdk1*	*Samd3*	*Try5*
Mfsd9	*Phyhd1*	*Scarf1*	*Tssk1*
Mgst2	*Pik3r3*	*Scrt1*	*Ttc8*
Mgst3	*Pkhd1l1*	*Sec14l2*	*Tubg2*
Mns1	*Plekha6*	*Sell*	*Tulp3*
Mocos	*Plekha8*	*Selm*	*Uevld*
Mpo	*Plin2*	*Sema4f*	*Vwa5a*
Mrpl14	*Plxdc1*	*Ska2*	*Wdr35*
Ms4a8a	*Poc1a*	*Slc7a4*	*Wdr54*
Mslnl	*Polr3gl*	*Slfn5*	*Wdr78*
Mthfd2l	*Ppap2a*	*Smtnl2*	*Wipi1*
Mvk	*Ppm1k*	*St3gal3*	*Zbp1*
Mxd4	*Ppp1r26*	*St6gal1*	*Zfp108*
Mybpc3	*Prkaca*	*Stk36*	*Zfp442*
Myh3	*Prkra*	*Stra13*	*Zfp449*
Myo7a	*Prlr*	*Stx7*	*Zfp551*
Naip2	*Psmb9*	*Tbc1d8*	*Zfp667*
Ndc80	*Ptgir*	*Tchh*	*Zfp72*
Nicn1	*Pxmp2*	*Tdgf1*	*Zfp946*
Nme4	*Pygl*	*Tdrd7*	*Zfp947*
Nusap1	*Rab19*	*Tert*	


A.
Group 4
C030034L19Rik
5830411N06Rik
Gpr97
Zfp112
Phf21b
Tlr1
Gm14446
Arnt2
Filip1l
Ret
Maf
4921525O09Rik
I830012O16Rik
Pde4a
Pou6f1
Dsg2
Mecom
Col13a1
Il17d
Bmp1
Itgax
Zmynd15
Slc2a10
Igf2bp1
Rpp25
Id3
Ccdc85c
Slc29a2
Khdc3
1700020L24Rik
Cxcr6
Xcr1
Mlana
Fhad1
Cilp2
Hoxd11
Gm2762
Cmklr1
Galr1
Mmp13
Rab27a
Tulp4
Pou2f2
Cdk5r1
Ccl4
AU021063
Utf1
Sema3f
Gm15612
D930028M14Rik
Cygb
Axin2
Wnk2
Podxl
Ccl9
Wnt3
Alpk2
Nfkbid
Pvrl1
Insm1
Srcin1
Kcna7
Cxcl16
Vash1
Shank3
Six1
Ccr3
Gjc1
Miat
Ngf
Tnf
Fgf18
Gnao1
Tead3
Slc30a3
Cd24a
Bcl2l1
Sytl3
Gm1045
Shank1
Slc32a1
Ccl20
Sox4
Kazn
Col4a4
Sult4a1
3.7
0
-3.7
Bulk
IFNγ- IL-10-
Bulk
IFNγ- IL-10-
IFNγ+ IL-10+
IFNγ+ IL-10-
IFNγ+ IL-10+
IFNγ+ IL-10-
Th1 + IL-27
Th1
Th1 + IL-27

B.



C.

Associated GO terms	Associated IPA Pathways	Genes Included
Chemokine activity, Chemokine receptor binding, Immune system process	Communication between innate & adaptive immune cells	*Id3, Maf, Nfkbid, Pou2f2, Arnt2, Wnt3, Mecom, Ccl4, Ccl9, Cxcl16, Ccl20*

D.

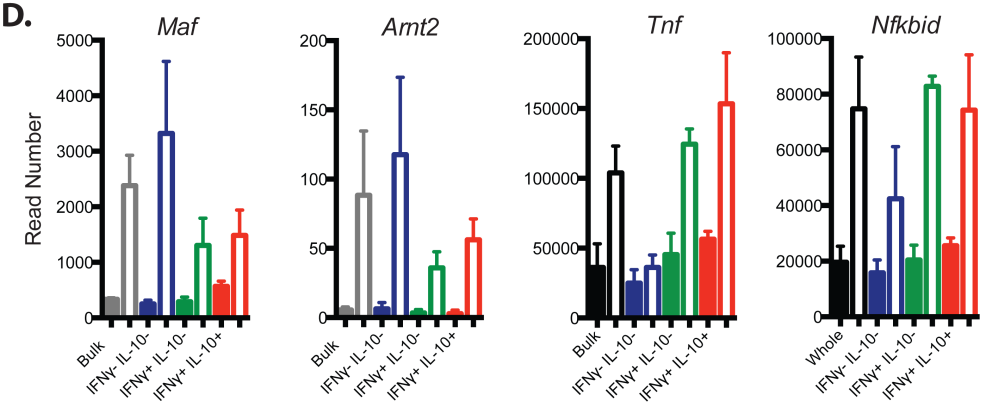


Figure 5B.9.1 Group 4: 86 genes upregulated in Th1 cells cultured with IL-27.

A. Genes in Group 4 from hierarchical clustering in Figure 5B.5. **B.** Expression profile of group. **C.** GO terms ($p<0.01$) and IPA pathways ($p<0.01$) with significant overlap, and examples of genes within the group. **D.** The read number of selected genes in each subpopulation.

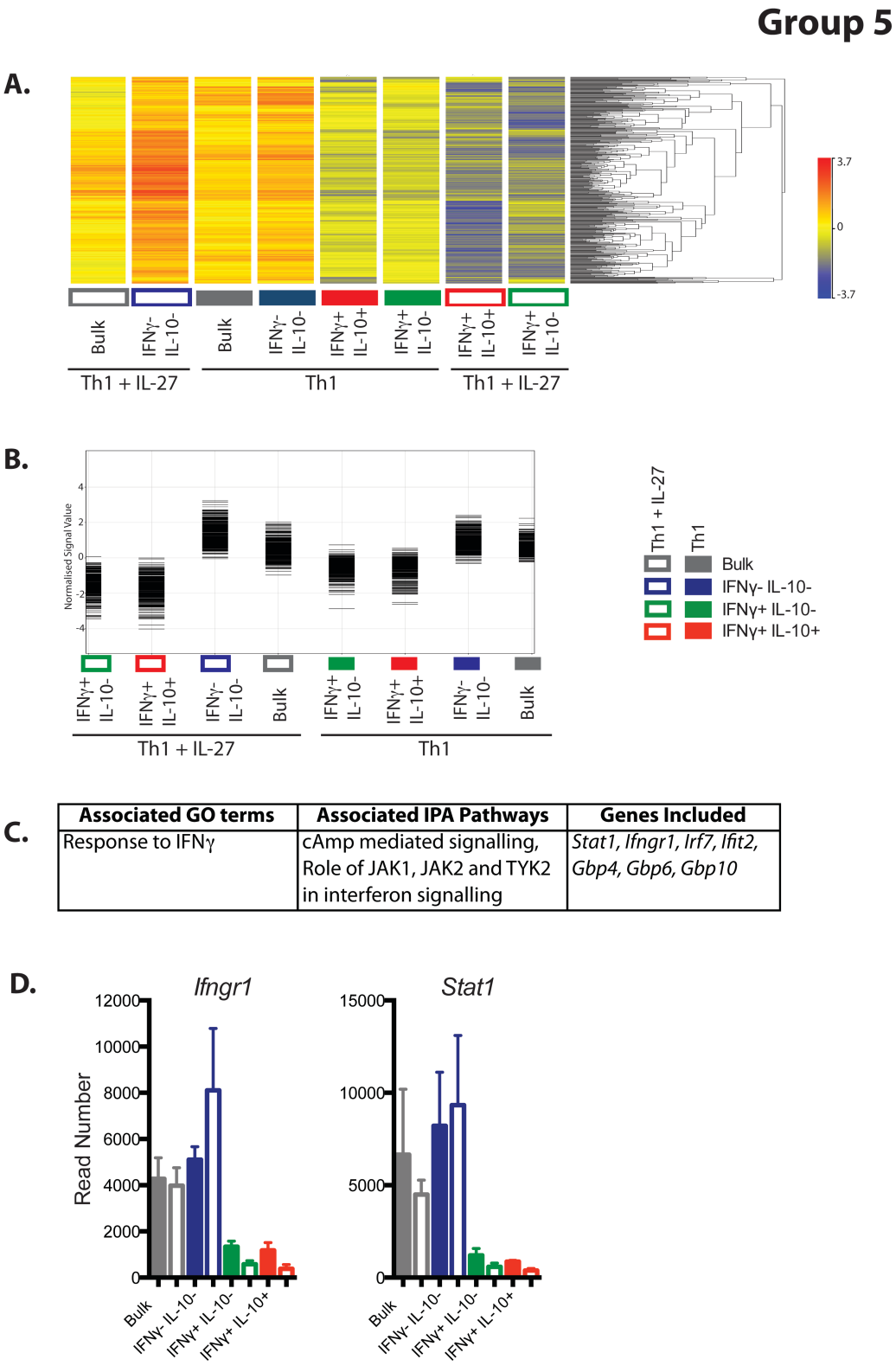
Figure 5B.9.2 Group 4: Network analysis of 86 genes upregulated in Th1 cells cultured with IL-27.

IPA network analysis of direct and indirect interactions between genes. Solid lines indicate direct interactions, the dashed lines indicate indirect interactions. Genes in red are those discussed.

Table 5B.9.1 Group 4: List of 86 genes upregulated in Th1 cells cultured with IL-27.

Genes highlighted in red are those also found in Group 2 of Table 5A.4.2.

1700020L24Rik	*Cxcr6*	*Kazn*	*Six1*
4921525O09Rik	*Cygb*	*Kcna7*	*Slc29a2*
5830411N06Rik	*D930028M14Rik*	*Khdc3*	*Slc2a10*
Alpk2	*Dsg2*	*Maf*	*Slc30a3*
Arnt2	*Fgf18*	*Mecom*	*Slc32a1*
AU021063	*Fhad1*	*Miat*	*Sox4*
Axin2	*Filip1l*	*Mlana*	*Srcin1*
Bcl2l1	*Galr1*	*Mmp13*	*Sult4a1*
Bmp1	*Gjc1*	*Nfkbid*	*Sytl3*
C030034L19Rik	*Gm1045*	*Ngf*	*Tead3*
Ccdc85c	*Gm14446*	*Pde4a*	*Tlr1*
Ccl20	*Gm15612*	*Phf21b*	*Tnf*
Ccl4	*Gm2762*	*Podxl*	*Tulp4*
Ccl9	*Gnao1*	*Pou2f2*	*Utf1*
Ccr3	*Gpr97*	*Pou6f1*	*Vash1*
Cd24a	*Hoxd11*	*Pvrl1*	*Wnk2*
Cdk5r1	*I830012O16Rik*	*Rab27a*	*Wnt3*
Cilp2	*Id3*	*Ret*	*Xcr1*
Cmklr1	*Igf2bp1*	*Rpp25*	*Zfp112*
Col13a1	*Il17d*	*Sema3f*	*Zmynd15*
Col4a4	*Insm1*	*Shank1*	
Cxcl16	*Itgax*	*Shank3*	



Associated GO terms	Associated IPA Pathways	Genes Included
Response to IFNγ	cAmp mediated signalling, Role of JAK1, JAK2 and TYK2 in interferon signalling	*Stat1, Ifngr1, Irf7, Ifit2, Gbp4, Gbp6, Gbp10*

Figure 5B.10.1 Group 5: 586 genes downregulated in cytokine producing subpopulations.

A. Genes in Group 5 from hierarchical clustering in Figure 5B.5. **B.** Expression profile of group. **C.** GO terms ($p<0.01$) and IPA pathways ($p<0.01$) with significant overlap, and examples of genes within the group. **D.** The read number of selected genes in each subpopulation.

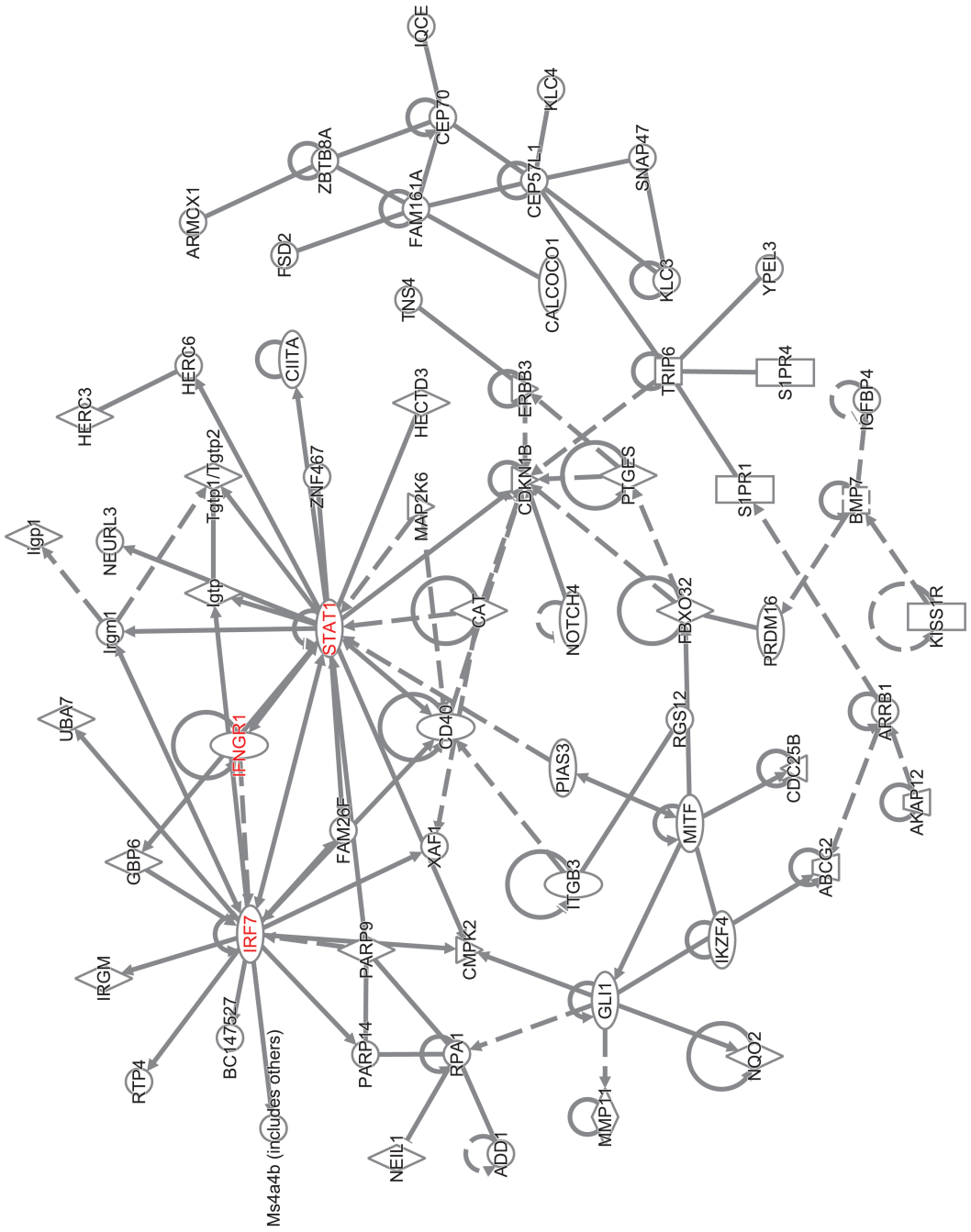


Figure 5B.10.2 Group 5: Network analysis of 586 genes downregulated in cytokine producing subpopulations.

IPA network analysis of direct and indirect interactions between genes. Solid lines indicate direct interactions, the dashed lines indicate indirect interactions. Genes in red are those discussed.

Table 5B.10.1 Group 5: List of 586 genes downregulated in cytokine producing subpopulations.

Genes highlighted in red are those also found in Group 2 of Table 5A.4.2.

1110058L19Rik	*Abhd6*	*Atp2a1*	*Cdc25b*
1300002E11Rik	*Abhd8*	*Avil*	*Cdc42ep1*
1600014C10Rik	*Ablim1*	*Azi1*	*Cdk19*
1600016N20Rik	*Acad10*	*B3gnt1*	*Cdkn1b*
1700019B03Rik	*Acad12*	*B3gnt5*	*Cela1*
1700020D05Rik	*Accs*	*Bambi-ps1*	*Cep164*
1700066J24Rik	*Acox3*	*Bank1*	*Cep57l1*
1810034E14Rik	*Acsf2*	*Bbs7*	*Cep70*
2010315B03Rik	*Acss1*	*BC029214*	*Cep97*
2410066E13Rik	*Acss2*	*BC051226*	*Cerk*
2510009E07Rik	*Adcy7*	*BC053749*	*Chst12*
2610008E11Rik	*Adcy9*	*BC064078*	*Ciita*
2610020H08Rik	*Add1*	*BC094916*	*Cirbp*
2810001G20Rik	*Aff3*	*BC147527*	*Clic3*
2810454H06Rik	*AI182371*	*Bcl7a*	*Clybl*
3010026O09Rik	*AI467606*	*Bmp7*	*Cmah*
3110062M04Rik	*AI847159*	*Bphl*	*Cmpk2*
3830408C21Rik	*Akap12*	*Brdt*	*Cnga1*
4833422C13Rik	*Akap17b*	*C1qtnf9*	*Cnp*
4921507P07Rik	*Aldh6a1*	*C230037L18Rik*	*Cnr2*
4930430F08Rik	*Alg14*	*Cabp4*	*Cpm*
4930441O14Rik	*Alg6*	*Cacna1h*	*Cradd*
4930451G09Rik	*Als2cl*	*Cacna2d4*	*Crebl2*
4930481A15Rik	*Amt*	*Cage1*	*Csgalnact1*
4930525G20Rik	*Ankrd12*	*Calcoco1*	*Ctdsp2*
4931431C16Rik	*Apol7b*	*Camkmt*	*Ctns*
4933404O12Rik	*Apol7e*	*Card14*	*Ctps2*
8430419L09Rik	*Appl2*	*Card6*	*Ctsf*
9530077C05Rik	*Arhgap25*	*Carns1*	*Cubn*
9930012K11Rik	*Arhgap4*	*Caskin2*	*Cxcr2*
9930111J21Rik1	*Arhgef18*	*Cat*	*Cxx1a*
A430033K04Rik	*Arl4c*	*Catsperg1*	*Cxx1b*
A430078G23Rik	*Arl5c*	*Ccdc125*	*Cxx1c*
A430088P11Rik	*Armc7*	*Ccdc176*	*Cybb*
A630023P12Rik	*Armcx1*	*Ccdc28a*	*Cyp2d22*
A930005H10Rik	*Arrb1*	*Ccdc69*	*Cyp2u1*
A930006K02Rik	*Arsa*	*Ccpg1*	*Cyp4f39*
AA474331	*Art2a-ps*	*Cd101*	*Cyth1*
Abca7	*Art2b*	*Cd19*	*D130017N08Rik*
Abcd1	*Art4*	*Cd200r4*	*D3Ertd751e*
Abcg2	*Asap3*	*Cd40*	*D630037F22Rik*
Abcg3	*Ascl4*	*Cd55*	*Dact3*
Abhd15	*Atg10*	*Cdadc1*	*Daf2*

Dand5
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Dars2
Ddb2
Ddx60
Degs2
Dguok
Dhrs7
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Entpd5
Epsti1
Erbb3
Ethe1
Evl
Fam101b
Fam105a
Fam135a
Fam161a
Fam189b
Fam26f
Fam49a
Fam63b
Fam65b
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Fam83f
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Fbxl20
Fbxo24
Fbxo32
Fcgr3
Fgl2
Flt4
Fmo5
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Fn3krp
Frmd8
Fry
Fsd2
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Haus4
Hectd3
Herc3
Herc6
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Hoxb4
Hs1bp3
Idh2
Ifit2
Ifngr1
Ift172
Igfbp4
Igtp
Iigp1
Ikzf4
Il16
Iqce
Irf7
Irgm1
Irgm2
Itfg3
Itga6
Itga7
Itgb3
Itpka
Itpr3
Jakmip1
Katnb1
Kbtbd11
Kbtbd3
Kcnip3
Kcnj8
Kctd12b
Kctd21
Kiss1r
Klc3
Klc4
Klf12
Klf2
Klhl36
Klhl4
Klhl6
Klrb1f
Lair1
Lats2
Ldlrap1
Lgmn
Lmbr1
Lrch4
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Lrrc56
Lypd6b
Lyrm7
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Map2k6
Map3k9
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Mitf
Mmp11
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Mrgpre
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Mut
Mxd1
Myo1f
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Nadsyn1
Napb
Nceh1
Ncmap
Ncoa7
Neil1
Neurl3
Nipa1
Nipal3
Nod1
Notch4
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Nsg2
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Oas1c
Oplah
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Pag1
Paqr7
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Parp14
Parp9
Patl2
Pcmtd2
Pcolce
Pctp
Pde11a
Pex11c
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Phf21a
Phkb
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Pigz
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Pitpnc1
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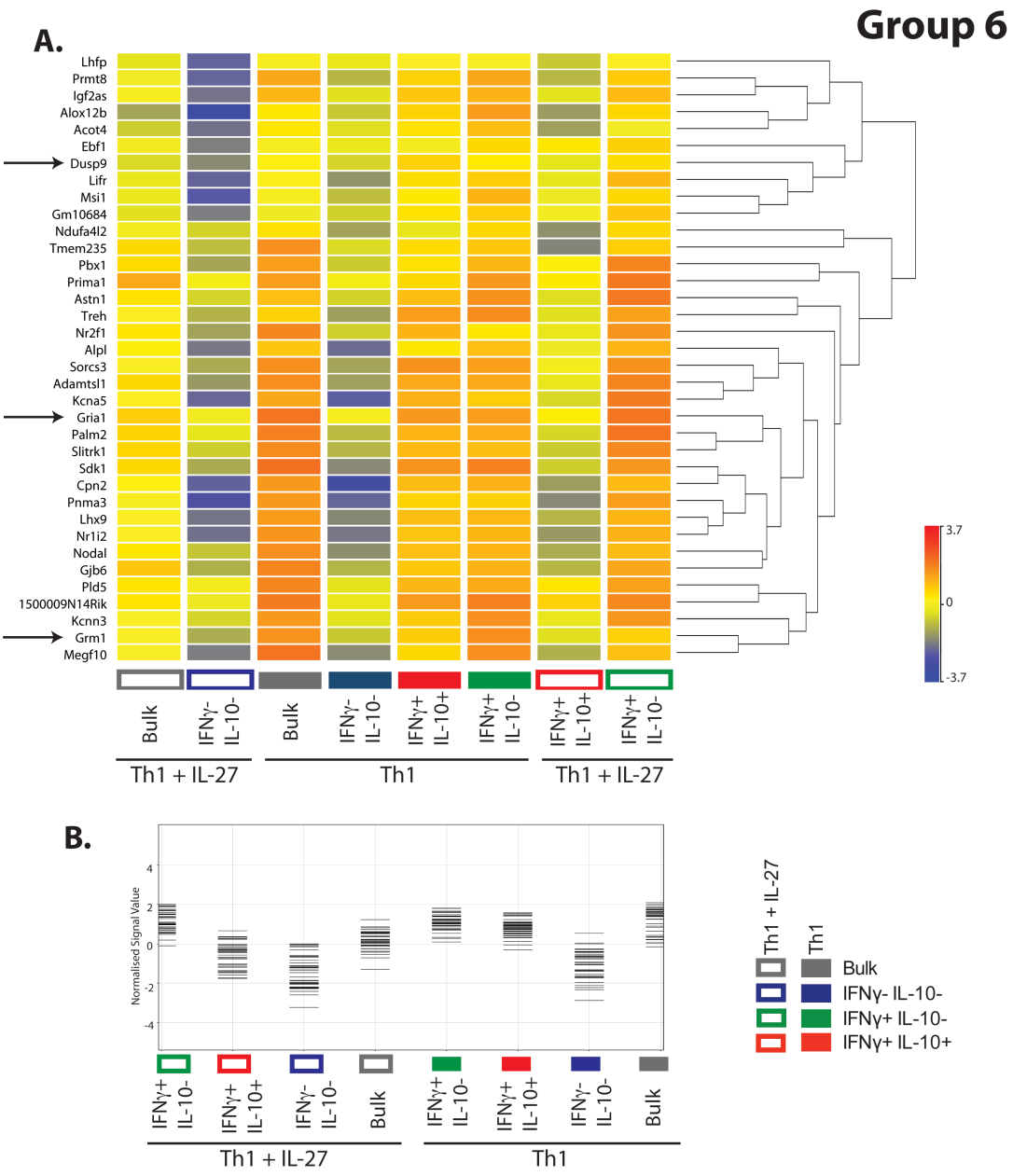
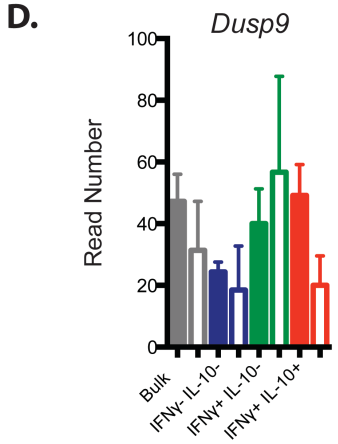
Ptges
Pxk
Pxmp4
Pydc3
Pydc4
Pyhin1
Qrfp
Rab37
Rab3d
Ranbp10
Ranbp3l
Rap1gap2
Rapgef4
Rasa3
Rasgrp2
Rassf2
Rgs12
Rgs14
Rnf125
Rnf13
Rnf32
Rnf39
Rpa1
Rpgrip1l
Rtbdn
Rtp4
Rundc3a
Ryr3
S1pr1
S1pr4
Sap25
Sarm1
Sbk1
Sbsn
Scd4
Scml4
Scn11a
Sec22c
Selplg
Sema6b
Senp7
Sepp1
Serp2
Sfxn5
Sh2d3c
Sh3bp1
Sh3d19
Sh3yl1
Shpk
Siae
Sidt1
Sipa1l3
Slc12a6
Slc12a9
Slc14a1
Slc1a1
Slc25a12
Slc25a27
Slc25a35
Slc25a45
Slc2a8
Slc35f5
Slc39a11
Slc41a3
Slc43a2
Slc44a2
Smyd4
Snap47
Snn
Sntb1
Sntb2
Snx32
Sorbs3
Sorcs2
Sp100
Spata6
Spo11
Ssbp2
Ssh2
St8sia1
St8sia6
Stat1
Stk38
Strc
Sult1a1
Suox
Susd3
Syt11
Tatdn3
Tbc1d10c
Tbc1d4
Tbx2
Tbx6
Tbxa2r
Tcf7
Tcn2
Tet1
Tex9
Tgtp1
Tgtp2
Tha1
Thap3
Themis2
Thra
Tia1
Tjp3
Tlr12
Tmco6
Tmem126b
Tmem140
Tmem220
Tmem241
Tmem40
Tmem63a
Tmem71
Tmem9
Tmem9b
Tmie
Tmprss13
Tnfaip8l2
Tnfrsf14
Tnfrsf26
Tnrc6b
Tns4
Tox3
Trim14
Trim34a
Trim47
Trim7
Trip6
Tspan32
Tspan9
Tssk4
Tstd1
Ttbk2
Ttc28
Ttc38
Ttll1
Ttyh3
Tubd1
Uba7
Ube2h
Ust
Vamp5
Wbscr27
Wdr19
Wfikkn2
Whsc1l1
Xaf1

Xkr5	*Zfp128*	*Zfp418*	*Zfp810*
Ypel2	*Zfp14*	*Zfp467*	*Zfp811*
Ypel3	*Zfp169*	*Zfp563*	*Zfp831*
Zbtb8a	*Zfp287*	*Zfp606*	*Zfp934*
Zc4h2	*Zfp30*	*Zfp637*	*Zfp945*
Zer1	*Zfp354c*	*Zfp69*	*Zscan2*
Zfand4	*Zfp383*	*Zfp78*	
Zfp101	*Zfp41*	*Zfp808*	



C.

Associated GO terms	Associated IPA Pathways	Genes Included
Glutamate receptor signalling	Glutamate receptor signalling, PPAR signalling, TGFβ signalling	*Grm1, Gria1, Dusp9, Nodal, Nr2f1*



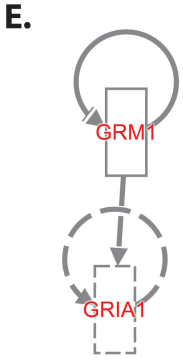
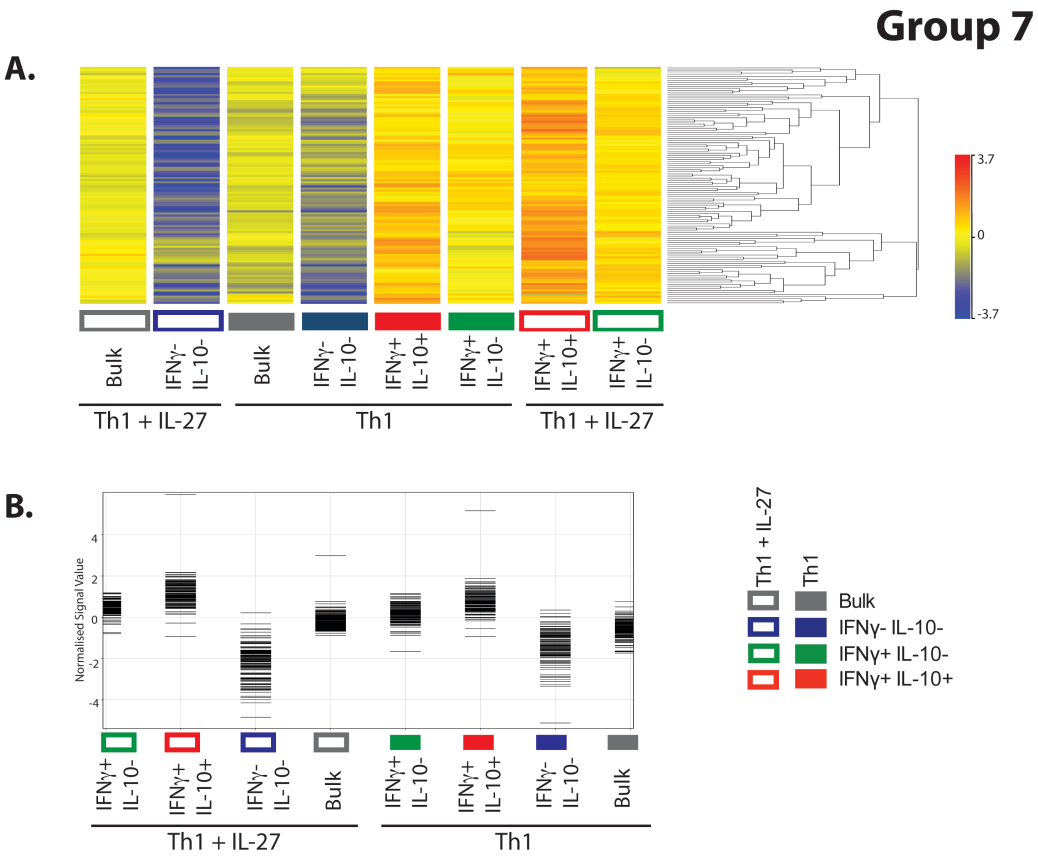


Figure 5B.11 Group 6: 37 genes upregulated in cytokine producing subpopulations. More pronounced in IFNγ+ IL-10- subpopulation

A. Genes in Group 6 from hierarchical clustering in Figure 5B.5, with gene names alongside. **B.** Expression profile of group. **C.** GO terms ($p<0.3$) and IPA pathways ($p<0.01$) with significant overlap, and examples of genes within the group. **D.** The read number of selected genes in each subpopulation. **E.** IPA network analysis of direct and indirect interactions between genes. Solid lines indicate direct interactions, the dashed lines indicate indirect interactions. Genes in red are those discussed.



C.

Associated GO terms	Associated IPA Pathways	Genes Included
Cytokine activity, Inflammatory response, Negative regulation of metabolism, Regulation of cytokine secretion, Regulation of IL-1 / IL-3 / IL-12 production	Role of cytokines in mediating communication between immune cells, IL-10 signalling, Communication between innate & adaptive immune cells, IL-12 / IL-6 signalling	*Il10, Ifng, Gzma, Il3, Il1a, Il23a, Il17f, Il22, Csf2, Cryba4, Ccl1, Ccl3, Cxcl13, Foxl2*

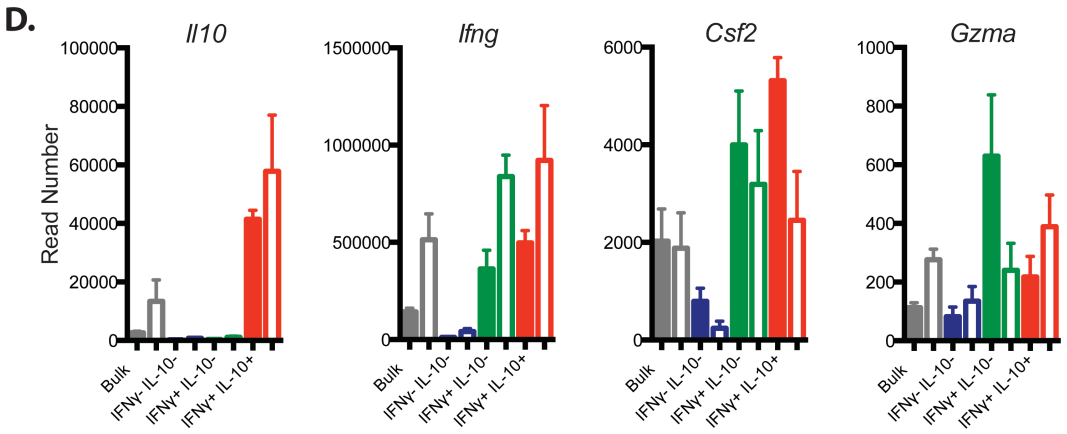


Figure 5B.12.1 Group 7: 124 genes upregulated in cytokine producing subpopulations. More pronounced in IFNγ+ IL-10+ subpopulation

A. Genes in Group 7 from hierarchical clustering in Figure 5B.5. **B.** Expression profile of group. **C.** GO terms ($p<0.01$) and IPA pathways ($p<0.01$) with significant overlap, and examples of genes within the group. **D.** The read number of selected genes in each subpopulation.

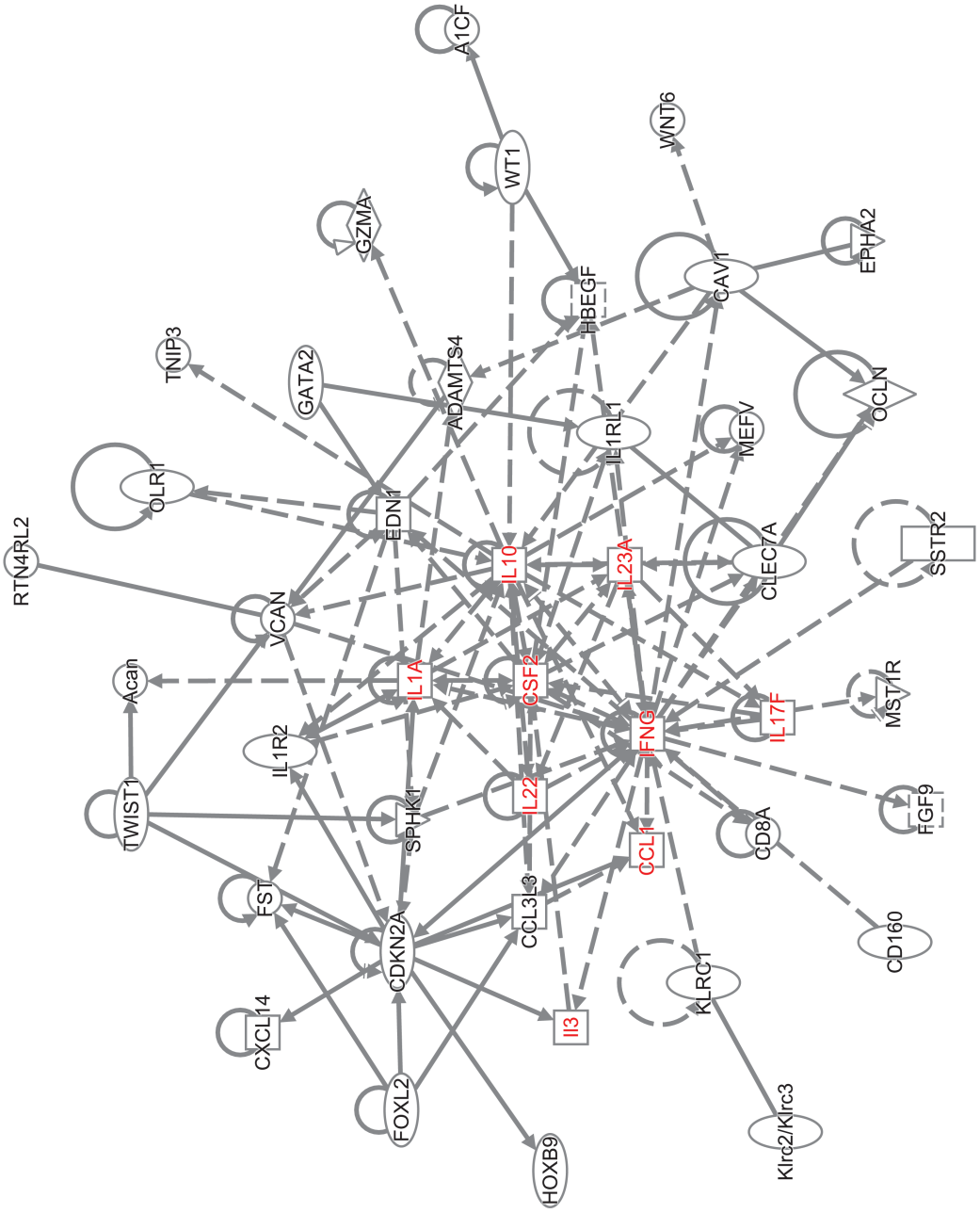


Figure 5B.12.2 Group 7: Network analysis of 124 genes upregulated in cytokine producing subpopulations. More pronounced in IFNγ+ IL-10+ subpopulation

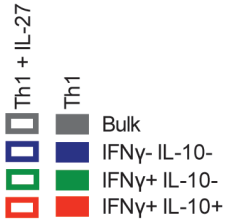
IPA network analysis of direct and indirect interactions between genes. Solid lines indicate direct interactions, the dashed lines indicate indirect interactions. Genes in red are those discussed.

Table 5B.12.1 Group 7: List of 124 genes upregulated in cytokine producing subpopulations. More pronounced in IFNγ+ IL-10+ subpopulations

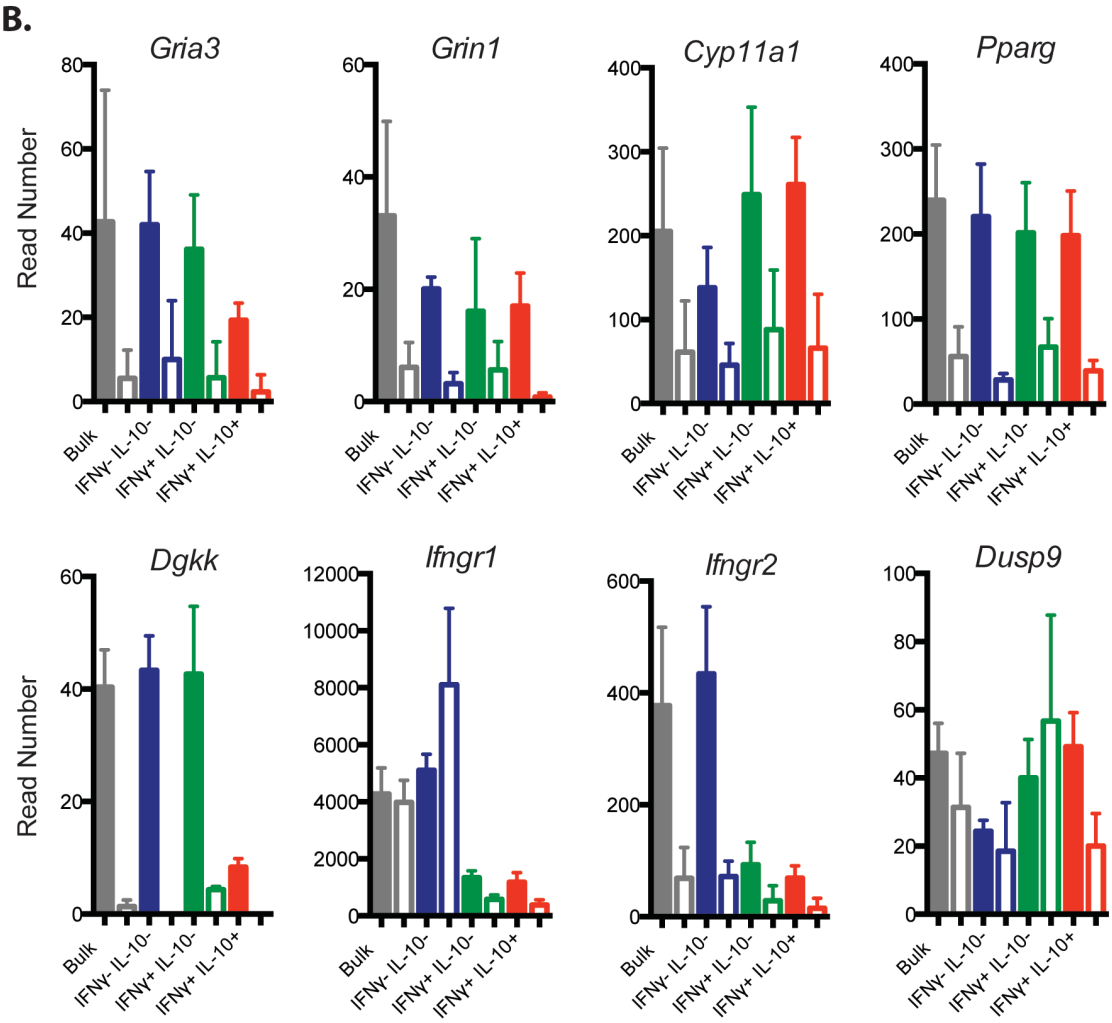
1700012B09Rik	*Cryba4*	*Gzma*	*Ocln*
1700016C15Rik	*Csf2*	*Hbegf*	*Olr1*
1810009J06Rik	*Cth*	*Hoxb2*	*Pdpn*
2310068J16Rik	*Cxcl14*	*Hoxb9*	*Pik3r6*
3300005D01Rik	*D930020B18Rik*	*Hrc*	*Plekhg4*
4930412O13Rik	*Dennd3*	*Idi2*	*Plscr2*
4933425H06Rik	*Dmxl2*	*Ifitm1*	*Rab44*
5730420D15Rik	*Dnahc11*	*Ifng*	*Rai14*
A1cf	*Drp2*	*Il10*	*Rhov*
Aanat	*E230029C05Rik*	*Il17f*	*Rrh*
Acan	*Edn1*	*Il1a*	*Rtn4rl2*
Adamts4	*Elfn1*	*Il1r2*	*Scrt2*
Akap6	*Epb4.1l4b*	*Il1rl1*	*Siglec5*
Arx	*Epb4.9*	*Il22*	*Slc45a3*
Astl	*Epha2*	*Il23a*	*Sowahb*
B4galt2	*Erv3*	*Il3*	*Sphk1*
Bpifc	*Etfb*	*Iltifb*	*Sstr2*
Card10	*Etnk2*	*Irx3*	*Timd2*
Cass4	*Fam13c*	*Ism1*	*Tmem178*
Cav1	*Fgf9*	*Kcnk10*	*Tmem200b*
Ccdc24	*Foxl2*	*Klrc1*	*Tnip3*
Ccl1	*Foxl2os*	*Klrc2*	*Trim66*
Ccl3	*Fst*	*Klrc3*	*Trpm6*
Cd160	*Gata2*	*Mcoln2*	*Twist1*
Cd70	*Gcnt2*	*Mcoln3*	*Unc80*
Cd8a	*Gcnt4*	*Mefv*	*Vcan*
Cd93	*Gm10389*	*Mmp20*	*Wnt11*
Cdkn2a	*Gm16516*	*Mst1r*	*Wnt6*
Cldnd2	*Gm2027*	*Myh6*	*Wnt9b*
Clec7a	*Gm20597*	*Nkg7*	*Wt1*
Col5a3	*Guca2b*	*Nkx2-4*	*Zfp57*

A.

	Seen in Bulk analysis	Only seen in subpopulation analysis
Ifngr1	Yes	Yes
Ifngr2		Yes
Cyp11a1	Yes	Yes
Gria3	Yes	Yes
Dgkk	Yes	Yes
Grin1		Yes
Pprag		Yes
Dusp9		Yes



B.



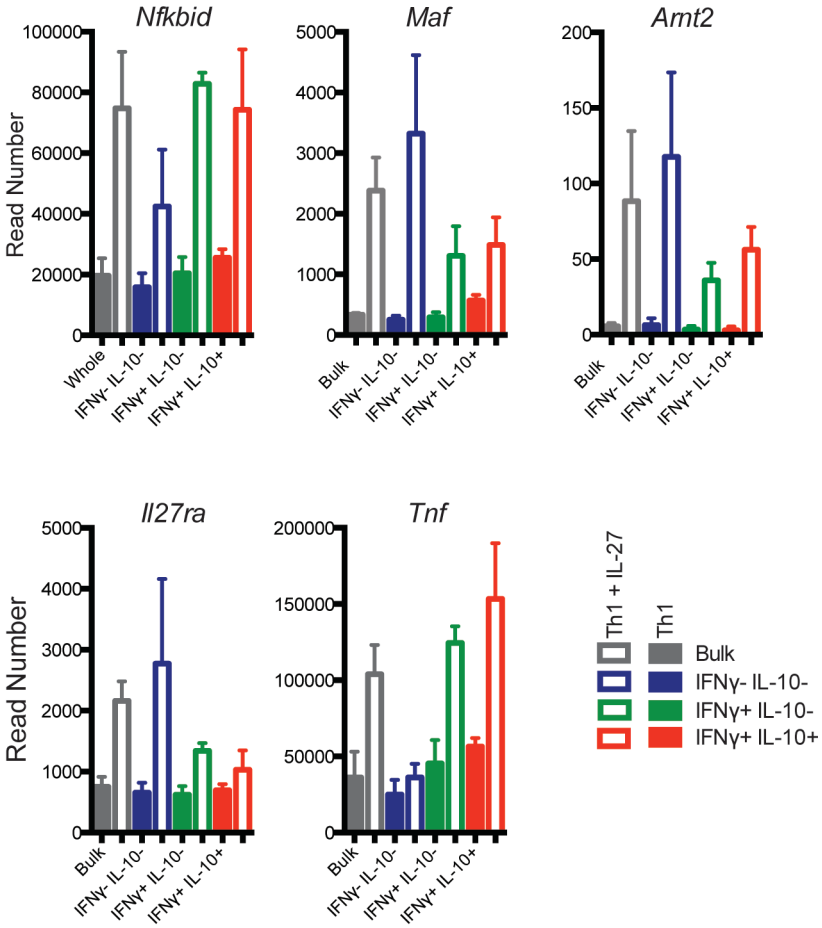
Discussion Figure 5.1 Genes of interest downregulated by IL-27

A. Table highlighting genes of interest found to be downregulated by IL-27 in Th1 cells. The first column highlights genes revealed in the analysis of the bulk unseparated Th1 and Th1 + IL-27 subsets. The second column highlights genes revealed in the analysis of different intracellular cytokine producing subpopulations within the Th1 and Th1 + IL-27 subsets. **B.** The read number of selected genes in each subpopulation.

A.

	Seen in Bulk analysis	Only seen in subpopulation analysis
Arnt2	Yes	Yes
Maf		Yes
Tnf		Yes
Nfkbid		Yes
Il27ra		Yes

B.



Discussion Figure 5.2 Genes of interest upregulated by IL-27

A. Table highlighting genes of interest found to be upregulated by IL-27 in Th1 cells. The first column highlights genes revealed in the analysis of the bulk unseparated Th1 and Th1 + IL-27 subsets. The second column highlights genes revealed in the analysis of different intracellular cytokine producing subpopulations within the Th1 and Th1 + IL-27 subsets. **B.** The read number of selected genes in each subpopulation.

Chapter 6. RNA-Seq analysis of differential gene expression in Th17 cells driven in the presence or absence of IL-2, and, in depth analysis of the different intracellular cytokine producing subpopulations within these Th17 + IL-2 and Th17 + anti-IL-2 subsets

Chapter 6 (A) RNA-Seq analysis of differential gene expression in Th17 cells driven in the presence or absence of IL-2

6.1 (A) Background

The mechanisms that regulate IL-10 production by Th17 cells are complex and remain unresolved. Th17 cells driven with TGFβ, rather than IL-1β, IL-6 and IL-23 have been shown to express higher levels of IL-10, and when driven with TGFβ and IL-6 alone Th17 cells express IL-17 and IL-10 but do not upregulate proinflammatory cytokines (Ghoreschi et al., 2010). However, the effects are variable and the cells in these conditions are highly heterogeneous (Kuchroo VK, International Congress of Immunology, 2013). When stimulated with IL-23 the cells express IL-17 and proinflammatory cytokines, including IFNγ, and no IL-10, leading to a pathogenic function (Ghoreschi et al., 2010; Lee et al., 2009b; McGeachy et al., 2007). As in Th1 cells, IL-27 can drive IL-10 from Th17 cells. IL-27 and IL-6 synergise with TGFβ to drive increased IL-10, but decreased IL-17, in Th17 cells, without affecting FoxP3 expression. The synergy of IL-27 and TGFβ has been suggested to drive IL-10 production via c-Maf, although this remains unclear (Xu et al., 2009). IL-27 driven IL-10 expression depends on STAT1 and STAT3 signalling, IL-6 driven IL-10 depends only on STAT3 signalling (Fitzgerald et al., 2007; Stumhofer et al., 2007).

There is a complicated and dynamic balancing process between Th17 and Treg differentiation, which is crucially regulated by the dose of TGFβ (Bettelli et al., 2006; Veldhoen et al., 2006). Low doses of TGFβ can synergise with IL-6 and IL-21 to initiate the development of Th17 responses, whereas high doses of TGFβ lead to the inhibition of IL-23 receptor expression and the induction of the Treg transcription factor FoxP3 (Awasthi and Kuchroo, 2009; Bettelli et al., 2006). IL-2, in conjunction with high levels of TGFβ, is thought to be essential for the differentiation of Treg cells *in vitro* (Davidson et al., 2007; Yamane and Paul, 2012) and the maintenance of Treg homeostasis *in vivo* (Fontenot et al., 2005). When culturing Th17 cells with IL-2 *in vitro* the expression of FoxP3 is increased (Laurence et al., 2007), STAT5

phosphorylation is increased (Zielinski et al., 2012), and the generation of Th17 cells and IL-17 expression is impaired. This is supported by findings that STAT5 represses RORγt and IL-17 upregulation (Laurence et al., 2007), and the inhibition of STAT5 or IL-2 results in greater IL-17 production (Zielinski et al., 2012). Furthermore, it has been demonstrated that IL-6, via STAT3, and IL-2, via STAT5, act reciprocally at the *Il17* locus to promote Th17 or Treg differentiation, respectively (Yang et al., 2011). However, alongside the role of IL-2 in suppressing Th17 cells, there is some evidence to suggest that IL-2 can drive transient IL-10 production by Th17 cells. It has been shown that during *S. aureus* infection IL-10 expression is transiently increased and IL-17 expression is decreased in Th17 cells, via what was postulated to be a STAT5 dependent, FoxP3 independent, mechanism (Zielinski et al., 2012).

IL-10 production by Th17 cells is variable and transient and the factors dominantly inducing IL-10 in Th17 cells are unclear; therefore we wanted to investigate the factors involved in driving IL-10 production from Th17 cells. We have found that IL-2 levels are crucial in driving two distinct forms of Th17 cell; addition of IL-2 results in increased IL-10, albeit alongside decreased IL-17, and blockade of IL-2 results in the disappearance of IL-10 alongside increased IL-17. We want to apply the method devised in Chapter 4 to perform RNA-Seq analysis on bulk cell populations of these ‘IL-10-’ or ‘IL-10+’ Th17 cells to address the effect of IL-2 on the transcriptional profile of Th17 cells and to illuminate some of the mechanisms behind IL-10 production in Th17 cells.

6.2 (A) Results

6.2.1 IL-10 production by Th17 cells increases, and IL-17 production decreases, upon culture with IL-2

IL-2 has been reported to repress Th17 cell generation and to promote Treg differentiation (Davidson et al., 2007; Laurence et al., 2007). Thus, IL-2 is thought to be important in balancing between the generation of RORγt+ Th17 cells and FoxP3+ Treg cells. Therefore, we wanted to investigate the role of IL-2 on Th17 differentiation and IL-10 production in our *in vitro* culture system. To test this, naïve CD4+CD62L+CD44loCD25- T cells were stimulated with anti-CD3 and anti-CD28, and cultured with TGFβ and IL-6 for the optimal 3 days (Chapter 3) (**Figure 6A.1 A**) in the presence of IL-2 (referred to as Th17 + IL-2 **Figure 6A.1 A**) or in the presence of anti-IL-2 (referred to as Th17 + anti-IL-2 **Figure 6A.1 A**); to neutralise any autocrine IL-2 produced by the Th17 cells. The presence of IL-2 did not effect the undetectable expression of IL-4 or IFNγ by the Th17 cells (**Figure 6A.1 A**), since cells were cultured in the presence of anti-IL-4 and anti-IFNγ. Treatment with anti-IL-2 lead to a slight increase in intracellular IL-2, which was also seen at the mRNA level, possibly due to the cells trying to compensate for the blocked IL-2 signalling in the extracellular environment. In keeping with the literature, treatment with anti-IL-2 significantly enhanced the production of IL-17 from Th17 cells (from 18.5% to 56%), although IL-17 was still produced by Th17 cells treated with IL-2 (21%) (**Figure 6A.1 A&B**). In addition, Th17 cells cultured in the presence of IL-2 produced increased amounts of IL-10 compared to the Th17 cells that only had endogenous IL-2 (from 4% to 14%) and from Th17 + anti-IL-2 cells as IL-10 was not observed in anti-IL-2 treated Th17 cells (**Figure 6A.1 A&B**). Furthermore, IL-10 was not only expressed by the subpopulations of cells that did not produce IL-17, but also by cells that did produce IL-17 (**Figure 6A.1 A**). None of the cells in either of the Th17 populations expressed FoxP3 and therefore were not IL-10 expressing Treg cells (data not shown). Therefore, the Th17 + IL-2 subset consisted of four subpopulations: IL-17- IL-10-, IL-17+ IL-10-, IL-17- IL-10+ and IL-17+ IL-10+. The Th17 + anti-IL-2 subset consisted of only two subpopulations: IL-17- IL-10- and IL-17+ IL-10-, and no IL-10+ subpopulations.

6.2.2 Quality control analysis of bulk Th17 + IL-2 and Th17 + anti-IL-2 RNA-Seq samples

6.2.2.1 Determining the optimal lower cut-off of reads

Prior to the identification of significantly differentially expressed genes in the Th17 + IL-2 and Th17 + anti-IL-2 samples, noise removal and quality control were carried out to ensure the integrity of the repeats. Once the RNA-Seq was performed, the transcripts were aligned, noise was removed (**Figure 6A.2 A**) and a quality control analysis was conducted to ensure the robustness of the experiments (**Figure 6A.2 B**). All the Th17 + IL-2 and Th17 + anti-IL-2 samples were pooled and the upper cut-off of reads for expression threshold was set at 253033.02 reads (where at least 1 out of 6 samples have values within cut-off), which was the maximal number of reads found for any gene. The number of entities passing expression thresholds with different lower-cut offs was assessed to determine where to set the lower cut-off (**Figure 6A.2 A**). As with in Chapter 5 the curve began to flatten at around 20 reads and the lower cut-off was set at 20 reads. Next we pooled the data from the three biological repeats of each subpopulation, and if any one gene in the three had a read count of above 20 then the entity would be included in the analysis. With the lower cut-off set at 20, the number of entities passing through each repeat was calculated (**Figure 6A.2 B**). The results revealed that each of the repeats had very tight error bars with all expressing between 11,200 – 11,500 entities. This showed that all of the repeats are comparable and are robust. Furthermore, as expected, we found that the mRNA expression profile of the cytokines *Il10* and *Il17a* mirrored the protein production profiles of the cells (**Figure 6A.2 C**). The Th17 + IL-2 cells expressed considerably more *Il10*, while the Th17 + anti-IL-2 cells expressed considerably more *Il17a.*

6.2.2.2 PCA and cluster analysis of bulk Th17 + IL-2 and Th17 + anti-IL-2

We performed a PCA on the bulk Th17 + IL-2 and Th17 + anti-IL-2 samples to further understand the variations in gene expression between the samples (**Figure 6A.3 A**). The repeats of the bulk Th17 + IL-2 and Th17 + anti-IL-2 subsets clustered away from each other. There was slightly more inter-repeat variability in the bulk Th17 + anti-IL-2 subset than between the bulk Th17 + IL-2 subset repeats, however this was minimal and

there were no obvious outliers in the data with the three repeats of each subpopulation clustering together.

To further determine the robustness of the samples and understand the variations in gene expression between the samples we performed unsupervised hierarchical clustering on the conditions. This clustering was performed on all 12,114 genes found within the samples (**Figure 6A.3 B**). As with the PCA, the dendrogram showed that the Th17 + IL-2 and Th17 + anti-IL-2 subsets clustered away from each other, suggesting that IL-2 has a distinct impact on the gene expression of Th17 cells. From this analysis we were assured that the repeats were robust and there were no outliers. Therefore, from this point forward, data from the three repeats was pooled. After pooling the data from the repeats we ran unsupervised hierarchical clustering on conditions and found that the bulk Th17 + IL-2 and bulk Th17 + anti-IL-2 subsets had visible differences in expression (**Figure 6A.3 C)**, however, overall their gene expression profiles looked very similar (as seen by the predominance of yellow on the heat map).

6.2.3 Hierarchical clustering separates the genes within the bulk Th17 + IL-2 and Th17 + anti-IL-2 cells into groups based on their expression profiles

Our data thus far demonstrates that culture of Th17 cells with IL-2 alters the transcriptional profile of Th17 cells. To further investigate the differential gene expression between the samples we applied statistical filtering to retain genes that were at least 3-fold up- or downregulated in at least one of the samples (repeat data pooled) vs. the baseline (median of all the samples). This resulted in a list of 861 genes that we then subjected to hierarchical clustering on entities and conditions (**Figure 6A.4.1 A**). The 861 gene set separated into two branches, 606 that were downregulated by IL-2 in Th17 cells (**Table 6A.4.1**), and 256 that were upregulated by IL-2 in Th17 cells (**Table 6A.4.2**). Therefore, the absence of IL-2 in Th17 cultures predominantly resulted in increased gene expression and the presence of IL-2 predominantly resulted in the downregulation of gene expression (**Figure 6A.4.1 A**).

To further investigate the genes in these two groups we assessed if they were significantly associated with any GO terms or IPA pathways. The genes in **Group 1** (**Table 6A.4.1**), which were downregulated by IL-2 in Th17 cells, were related to

retinoic acid metabolic process, cell adhesion, regulation of phosphate metabolic process, FXR/RXR activation, P2Y purigenic receptor signalling and MIF regulation of innate immunity (JNK/AP1 signalling) (**Figure 6A.4.1 B**). Genes of interest included *Cyp26a1* and *Cyp26b1*, which encode enzymes that degrade retinoic acid (Rhinn and Dolle, 2012), and also various factors associated with ERK / AP-1 signalling were present, including *Egr1, Egr3, Fos, Fosb* and *Twist1*, and the cytokines *Il2, Il17a* and *Il23a* (**Figure 6A.4.1 B**). Network analysis revealed that many of these genes directly interact with one another, with FOS, TWIST1 and EGR1 playing central roles in this network (**Figure 6A.4.2**).

The genes in **Group 2** (**Table 6A.4.2**), which were upregulated by IL-2 in Th17 cells, were related to cytokine receptor activity, immune system effector process, cell migration, inflammatory and immune responses, CTLA4, IL-12 & IL-10 signalling, T helper cell differentiation and CD28 signalling in T helper cells (**Figure 6A.4.1 C**). Genes of interest included the cytokine receptors *Il10ra, IL12rb1* and *Il12rb2*, the chemokine receptors *Cxcr1*, *Ccr2*, *Ccr4* and *Ccr5*, the cell surface molecules *Cd80* and *Cd86*, the transcription factors *Maf* and *Prdm1*, and as expected *Il10* (**Figure 6A.4.1 C**); showing the dependence of these genes on IL-2. Most of these genes are interrelated at the network level, stemming from the core nodes of MAF and PRDM1 (**Figure 6A.4.3**).

This analysis revealed many interesting differences between Th17 cells cultured in the presence of IL-2 or anti-IL-2. Of particular interest is the finding that IL-2 may downregulate the expression of molecules involved in retinoic acid metabolism and the expression of genes involved in ERK / AP-1 signalling. *Prdm1* and *Maf* have been associated with IL-10 regulation in Th1 cells (Neumann et al., 2014), and therefore IL-2 may drive *Il10* expression in Th17 cells via this pathway. However, as analysis of the different cytokine producing Th1 cell subpopulations revealed many more pathways involved in IL-27 driven Th1 cell differentiation and IL-10 production, we wanted to perform a similar analysis on the different intracellular producing subpopulations within the Th17 + IL-2 and Th17 + anti-IL-2 subsets to investigate in greater depth the effect of IL-2 on *Il10* gene expression and the transcriptional profiles of Th17 cells, and uncover novel mechanisms or factors that may be involved in the regulation of *Il10* gene expression.

Chapter 6 (B) In depth analysis of the different intracellular cytokine producing subpopulations within these Th17 + IL-2 and Th17 + anti-IL-2 subsets

We next asked the question whether the separation of subpopulations within the heterogeneous Th17 + IL-2 and Th17 + anti-IL-2 bulk populations could reveal additional novel factors involved in IL-2 driven Th17 cell differentiation and IL-10 production; which may be undetectable due to the potential masking by the large IL-17- IL-10- subpopulation of cells within the bulk cultures.

6.3 (B) Background

As with other Th subsets, Th17 cultures are heterogeneous and contain distinct subpopulations, those that don't make cytokines and those that do produce cytokines. In support of this, it has been found that only single cell RNA-Seq can reveal regulatory factors involved in the 'non-pathogenic' IL-10+ phenotype of Th17 cells, and single cell RNA-Seq of Th17 cells has highlighted the vast amount of heterogeneity within the Th17 population (Kuchroo VK, International Congress of Immunology, 2013). Our work separating different intracellular cytokine producing subpopulations within Th1 and Th1 + IL-27 subsets has highlighted significant transcriptional differences between these subpopulations. In the first section of this chapter we optimised a system for culturing IL-10- and IL-10+ Th17 cells *in vitro* with anti-IL-2 and IL-2, respectively, and analysed the bulk population of these cells. Now, to determine the transcriptional profiles of the different intracellular cytokine producing cells from within the heterogeneous populations of Th17 + IL-2 and Th17 + anti-IL-2 cells, we again used the approach of analysing different intracellular cytokine producing subpopulations by RNA-Seq to study Th17 cells and the effects of IL-2 signalling on Th17 cell IL-10 production.

6.4 (B) Results

6.4.1 Separating different intracellular cytokine producing subpopulations of Th17 cells

As highlighted in **Figure 6A.1**, the Th17 + IL-2 and Th17 + anti-IL-2 subsets are heterogeneous populations of cells with regard to protein production. Therefore, we were interested in applying the technique developed in Chapter 4 to separate the different intracellular cytokine producing subpopulations within the Th17 + IL-2 and Th17 + anti-IL-2 subsets. We elected to carry out RNA-Seq analysis on the Th17 + IL-2 and Th17 + anti-IL-2 subsets as we were interested in genes affected by IL-2 and genes associated with the different intracellular cytokine producing subpopulations. We separated these subsets into different intracellular cytokine producing subpopulations based on the production of IL-17 and IL-10 (**Figure 6B.1**). The mRNA from the four different subpopulations within the Th17 + IL-2 subset was extracted for RNA-Seq analysis: IL-17- IL-10-, IL-17+ IL-10-, IL-17- IL-10+ and IL-17+ IL-10+. Alongside the mRNA was isolated from the two different subpopulations within the Th17 + anti-IL-2 subset: IL-17- IL-10- and IL-17+ IL-10-.

The separated subpopulations represented similar percentages of the bulk Th17 + IL-2 and Th17 + anti-IL-2 populations in the three repeat experiments (**Figure 6B.1**). In the Th17 + anti-IL-2 subset, the IL-17- IL-10- subpopulation represented on average of 36% of the bulk, the IL-17+ IL-10- subpopulation 50.5%. Therefore the major proportion of this subset was the IL-17 producing cells (**Figure 6B.1 A**). In the Th17 + IL-2 subset, the IL-17- IL-10- subpopulation represented on average 55.3% of the bulk, the IL-17+ IL-10- subpopulation 14.8%, the IL-17+ IL-10+ subpopulation 7.8%, and the IL-17- IL-10+ subpopulation 13.1% (**Figure 6B.1 B**). The IL-17+ IL-10- subpopulation showed a decrease as a result of IL-2 signalling, while the IL-17- IL-10+ and IL-17+ IL-10+ subpopulations were only detected as a result of IL-2. The total cytokine producing subpopulations consisted of about 35.7% of the bulk population (**Figure 6B.1 B**).

6.4.2 Quality control analysis of Th17 + IL-2 and Th17 + anti-IL-2 subpopulation RNA-Seq samples

6.4.2.1 Determining the optimal lower cut-off of reads

As with the analysis of the bulk Th17 + IL-2 and Th17 + anti-IL-2 samples we performed noise removal and quality control to ensure the integrity of the repeats. Once the RNA-Seq was performed, the transcripts were aligned, noise was removed (**Figure 6B.2 A**) and a quality control analysis was conducted to ensure the robustness of the experiments (**Figure 6B.2 B**). All the Th17 + IL-2 and Th17 + anti-IL-2 samples were pooled and the upper cut-off of reads for expression threshold was set at 323885.938 reads (where at least 1 out of 24 samples had values within the cut-off), which was the maximal number of reads found for any gene. The number of entities passing expression thresholds with different lower-cut offs was assessed to determine where to set the lower cut-off (**Figure 6B.2 A**). As with the bulk Th17 + IL-2 and Th17 + anti-IL-2 samples, the curve began to flatten at around 20 reads and the lower cut-off was set at 20 reads. Next, we pooled the data from the three biological repeats of each subpopulation, and with the lower cut-off set at 20, the number of entities passing through each repeat was calculated (**Figure 6B.2 B**). The results revealed that each of the repeats expressed between 11,000 – 12,000 entities. This showed that all of the repeats are comparable and are robust.

6.4.2.2 PCA and cluster analysis of Th17 + IL-2 and Th17 + anti-IL-2 subpopulations

We performed PCA on the Th17 + IL-2 and Th17 + anti-IL-2 samples to further understand the variations in gene expression between the samples (**Figure 6B.3 A**). For the Th17 + IL-2 and Th17 + anti-IL-2 samples, the PCA showed that the greatest variation in the data was due to the presence or absence of IL-2 in the culture. The second component split the Th17 + IL-2 cells based on the production of IL-10; the IL-10 producing subpopulations within the Th17 + IL-2 subset clustered close to one another and away from the bulk, IL-17+ IL-10- and IL-17- IL-10- subpopulations (**Figure 6B.3 A**). The bulk Th17 + IL-2 and Th17 + anti-IL-2 cells fell between the IL-17- IL-10- and IL-17+ IL-10- subpopulations. There was a large amount of inter-

experimental variability between the samples of the Th17 + anti-IL-2 subset, however there were no obvious outliers in the data (**Figure 6B.3 A**).

To further determine the robustness of the samples and understand the variations in gene expression between the subpopulations we performed unsupervised hierarchical clustering on the conditions. This clustering was performed on all 12,536 genes found within the samples (**Figure 6B.3 B**). As with the PCA, the dendrogram showed the Th17 + IL-2 and Th17 + anti-IL-2 subsets cluster away from each other. Suggesting that IL-2 has a distinct impact on the gene expression of Th17 cells. The branch lengths of the dendrogram are representative of similarity, and compactness of the branches between the subpopulations within the Th17 + anti-IL-2 subset suggested that all the subpopulations within this subset were very similar. This may be due to the inter-group variability between the repeats, seen in the PCA (**Figure 6B.3 A**), causing overlap between the expression profiles of these samples. The Th17 + anti-IL-2 bulk, IL-17+ IL-10- and IL-17- IL-10- subpopulations were interspersed, further suggesting there were only minor differences between the subpopulations in this subset driven in the absence of IL-2. Within the Th17 + IL-2 subset the subpopulations separated into two main groups; repeats of the two IL-10+ subpopulations clustered together, repeats of the bulk and IL-17- IL-10- subpopulations clustered together, and the IL-17+ IL-10- repeats were interspersed between these two groups (**Figure 6B.3 B**). These data show that the presence or absence of IL-2, and the production of cytokines, both have distinct effects on gene expression.

6.4.2.3 Different intracellular cytokine producing subpopulations within the Th17 + IL-2 and Th17 + anti-IL-2 have different transcriptional profiles

After pooling data from the three repeats we ran unsupervised hierarchical clustering on conditions. Again, we found that the major factor separating the subpopulations was the presence or absence of IL-2 in the culture (**Figure 6B.4 A**). Furthermore, these differences cannot be attributed to differences in the expression of the Th17 master regulator *Rorc*; as all of the subpopulations in both the Th17 + IL-2 and Th17 + anti-IL-2 subsets expressed similar levels of this gene (**Figure 6B.4 B**). Moreover, the compactness of the branches between the subpopulations within the Th17 + anti-IL-2 subset again suggested that the subpopulations within this subset were very similar,

though the bulk and IL-17+ IL-10- subpopulations looked to be slightly more closely related (**Figure 6B.4 A**). Within the Th17 + IL-2 subset, the IL-10 producing subpopulations clustered together and separately from the other subpopulations, the bulk and IL-17- IL-10- subpopulations clustered together. The IL-17+ IL-10- subpopulation was more closely related to the bulk and IL-17- IL-10- subpopulations, but lay between the two groups. This suggested that a major factor separating the Th17 + IL-2 subpopulations was the production of IL-10 (**Figure 6B.4**).

6.4.3 Hierarchical clustering separates the Th17 + IL-2 and Th17 + anti-IL-2 subpopulations into groups based on their gene expression profiles

Our data thus far demonstrates that culture of Th17 cells with IL-2, or the production of cytokines, alters the transcriptional profile of Th17 cells. To further investigate the differential gene expression between the samples we applied statistical filtering to retain genes that were at least 3-fold up- or downregulated in at least one of the samples (repeat data pooled) vs. the baseline (median of all the samples). This resulted in a list of 1098 genes that we then subjected to hierarchical clustering on entities and conditions (**Figure 6B.5**).

The 1098 gene set separated into two branches; the Th17 + IL-2 subpopulations clustered into one group and the Th17 + anti-IL-2 subpopulations clustered to a different group. There were two clear groups of genes with different transcriptional profiles within this 1098 set of genes:

- Group 1: 749 genes downregulated by IL-2 in Th17 cells.
- Group 2: 349 genes upregulated by IL-2 in Th17 cells.

6.4.3.1 Genes downregulated by IL-2 in Th17 cells

Group 1 consisted of 749 genes (**Table 6B.6.1**) that were downregulated in Th17 cells cultured in the presence of IL-2 (**Figure 6B.6.1 A&B**). The results from this group were similar to those seen for **Group 1** in the analysis of the bulk unseparated Th17 + IL-2 and Th17 + anti-IL-2 cells (**Figure 6A.4.1**). Pathways and terms related to this list of genes included retinoic acid metabolic process, cell differentiation and JNK/AP1

signalling (**Figure 6B.6.1 C**). *Cyp26a1* and *Cyp26b1*, are again in this list, alongside various factors associated with ERK and AP-1 signalling, including *Egr3, Fos, Fosb* and *Twist1*. Furthermore, factors not seen in the analysis of the bulk unseparated Th17 + IL-2 and Th17 + anti-IL-2 cells were revealed, including the ERK associated factors *Twist2* and *Mapk10*, which encodes the c-Jun N-terminal Kinase JNK3 (**Figure 6B.6.1 C**). Though we see similar results to the analysis of the bulk unseparated Th17 + IL-2 and Th17 + anti-IL-2 cells, by performing analysis on the different subpopulations we gain greater insight into the expression profiles of these genes. *Fos, Fosb* and *Egr3* follow a similar expression pattern to each other; the bulk Th17 + anti-IL-2 and IL-17+ IL-10- subpopulation express similar levels while the number of reads in the IL-17- IL-10- subpopulation was much higher (**Figure 6B.6.1 D**). None of the Th17 + IL-2 subpopulations expressed significant amounts of these genes (**Figure 6B.6.1 D**), suggesting that IL-2 suppresses their expression. Network analysis of the genes in this list revealed a close interaction with FOS, FOSB and FOSL1 (**Figure 6B.6.2**). Furthermore, MAPK10 interacts with two genes, MAPK8IP1 & 2, that are known to interact with another c-Jun N-terminal Kinase: JNK1 (**Figure 6B.6.2**). 512 of the genes in this list (**Table 6B.6.1** highlighted in red) were also seen to be downregulated in the analysis of the bulk unseparated Th17 + IL-2 and Th17 + anti-IL-2 cells (Group 1, **Table 6A.4.1**). However, a further 237 genes in this list were not seen to be downregulated by IL-2 in the analysis of the bulk unseparated Th17 + IL-2 and Th17 + anti-IL-2 cells, showing that analysis of the separated subpopulations reveals more factors.

6.4.3.2 Genes upregulated by IL-2 in Th17 cells

Group 2 consisted of 349 genes (**Table 6B.71**) that were upregulated in Th17 cells cultured in the presence of IL-2 (**Figure 6B.7.1 A&B**). Again, the results from this group were similar to those seen for **Group 2** in the analysis of the bulk unseparated Th17 + IL-2 and Th17 + anti-IL-2 cells (**Figure 6A.4.1**). Multiple pathways involved in cytokine signalling, immune responses and T cell signalling were associated with this list of genes (**Figure 6B.7.1 C**). However, additional cytokine, cytokine receptor and chemokine receptor genes were revealed, including *Il10, Ifng, Il24, Il1a, Csf2, Il12rb1, Il12rb2, Il10r, Il7r, Ccr2, Ccr4* and *Ccr5* (**Figure 6B.7.1 C**). Furthermore, expression of the surface protein CD86 was upregulated by IL-2 (**Figure 6B.7.1 C**), this is in

keeping with the literature that has shown that IL-2 upregulates CD86 expression in human CD4+ T cells (Paine et al., 2012).

The expression profile of *Il10* matched the protein production profile of the cells, with undetectable *Il10* expression in the Th17 + anti-IL-2 subpopulations and very little expression of *Il10* in the non-IL-10 producing subpopulations of the Th17 + IL-2 subset (**Figure 6B.7.1 D**). *Il10* was highly expressed in the Th17 + IL-2 IL-17- IL-10+ and IL-17+ IL-10+ subpopulations: to the greatest extent in the IL-17+ IL-10+ subpopulation (**Figure 6B.7.1 D**). The amount of *Il10* expressed in the bulk Th17 + IL-2 subset was dwarfed by the amounts seen in the IL-17- IL-10+ and IL-17+ IL-10+ subpopulations. However, *Il10ra* expression was upregulated in all of the Th17 + IL-2 subpopulations, suggesting the IL-10 produced by only 21% of the cells could feedback on all of the subpopulations (**Figure 6B.7.1 D**).

Maf was also highly expressed in the Th17 + IL-2 subset compared to the Th17 + anti-IL-2 subset (**Figure 6B.7.1 D**). c-Maf clearly required IL-2 for its expression since this was abrogated when anti-IL-2 had been added to the cultures. Network analysis of this list of genes highlighted two core node genes, EGFR and ESR1. MAF, IL10 and the IL10RA interacted with one another and MAF also interacted with IL12RB1. IL12RB2 and IFNγ were also found within this netwrok. Furthermore, two AhR targets, AHRR and CYP1B1 were found within this network, which was not seen in the bulk comparison (**Figure 6B.7.2**). 1 of the genes in this list (**Table 6B.7.1** highlighted in red) was also seen to be downregulated by IL-2 in the analysis of the bulk unseparated Th17 + IL-2 and Th17 + anti-IL-2 cells (Group 1, **Table 6A.4.1**). 210 of the genes in this list (**Table 6B.7.1** highlighted in blue) were also seen to be upregulated by IL-2 in the analysis of the bulk unseparated Th17 + IL-2 and Th17 + anti-IL-2 cells (Group 2, **Table 6A.4.2**). However, a further 138 genes in this list were not seen to be upregulated by IL-2 in the analysis of the bulk unseparated Th17 + IL-2 and Th17 + anti-IL-2 cells, showing that analysis of the separated subpopulations reveals more factors.

6.4.4 Hierarchical clustering separates the Th17 + IL-2 intracellular cytokine producing subpopulations into 2 major groups based on their gene expression profiles

The dramatic difference in the gene expression profiles of the Th17 + IL-2 and Th17 + anti-IL-2 subsets meant that there was little difference between the results of analysis of the bulk subsets (**Figure 6A.4.1**) and analysis of the different subpopulations (**Figure 6B.5, 6B.6 & 6B.7**). Therefore we saw a loss of resolution of the transcriptional differences between the subpopulations within the subsets. Consequently, to investigate transcriptional differences between the subpopulations of the Th17 + IL-2 and Th17 + anti-IL-2 subsets, we chose to analyse each of them in more depth separately.

To further investigate the differential gene expression between the subpopulations within the Th17 + IL-2 subset we applied statistical filtering to retain genes that were at least 2-fold up- or downregulated in at least one of the samples (repeat data pooled) vs. the baseline (median of all the samples). This resulted in a list of 483 genes that we then subjected to hierarchical clustering on entities and conditions (**Figure 6B.8**). The 483 gene set separated into two major branches; the IL-17+ IL-10-, IL-17- IL-10+ and IL-17+ IL-10+ cytokine producing subpopulations clustered into one group and the IL-17- IL-10- subpopulation clustered with the bulk Th17 + IL-2 cells. There were two clear groups of genes with different transcriptional profiles within this 483 set of genes:

- Group 1: 313 genes downregulated in the cytokine producing subpopulations of the Th17 + IL-2 cells.
- Group 2: 170 genes upregulated in the cytokine producing subpopulations of the Th17 + IL-2 cells.

6.4.4.1 Genes that are downregulated in the cytokine producing subpopulations of the Th17 + IL-2 subset

Group 1 consisted of 313 genes that were downregulated in the cytokine producing subpopulations of the Th17 + IL-2 cells (**Figure 6B.9.1 A&B**). Some of these genes were downregulated in the IL-17+ IL-10- and IL-17+ IL-10+ subpopulations (**Figure**

6B.9.1 A, List 1, **Table 6B.9.1**). Some of these genes were downregulated in the IL-17- IL-10+ and IL-17+ IL-10+ subpopulations (**Figure 6B.9.1 A,** List 2, **Table 6B.9.2**). Some of these genes were only downregulated in the IL-17- IL-10+ subpopulation (**Figure 6B.9.1 A,** List 3, **Table 6B.9.3**). Further analysis of List 1 (**Table 6B.9.1**) and List 3 (**Table 6B.9.3**) revealed no significant GO terms or IPA pathways associated with these gene sets. However, further analysis of List 2 (**Table 6B.9.2**) revealed pathways and terms including cellular metabolic processes and cell cycle (**Figure 6B.9.2 A**).

Genes in List 2 (**Table 6B.9.2**) included *Bard1, Cdkn1, Ccnb1* and *FoxM1* (**Figure 6B.9.2 A**). BARD1 and FoxM1 have both been associated with cell cycle. CDK1 and CCNB1 are downstream of FoxM1. In particular, FoxM1 has been suggested to be a master cell cycle regulator in T cells (Xue et al., 2010). The expression of *Foxm1* was highly downregulated in the IL-17- IL-10+ and IL-17+ IL-10+ subpopulations of the Th17 + IL-2 subset. However, its expression was high in the bulk, IL-17- IL-10- and IL-17+ IL-10- subpopulations (**Figure 6B.9.2 B**). The expression of *Cdkn1* and *Ccnb1* mirrored the *Foxm1* expression profile (**Figure 6B.9.2 B**). This suggests cell cycle regulators were specifically downregulated in the IL-10 producing subpopulations of the Th17 + IL-2 subset. Network analysis further revealed the central role FOXM1 played in the interaction of genes within this list (**Figure 6B.9.3**), which also contained many cell cycle regulating genes; including the cyclins CCNA2 and CCNB2, and the cell division cycle associated genes CDCA2, CDCA5 and CDC25C.

6.4.4.2 Genes that are upregulated in the cytokine producing subpopulations of the Th17 + IL-2 subset

Group 2 consists of 170 genes (**Table 6B.10.1**) that were upregulated in the cytokine producing subpopulations of the Th17 + IL-2 cells (**Figure 6B.10.1 A&B**). Most of the genes were upregulated in the IL-17+ IL-10+ subpopulation of cells. However, only some of the genes are upregulated in the IL-17+ IL-10- subpopulation (**Figure 6B.10.1 A**, List 1 & 3) or the IL-17- IL-10+ subpopulation (**Figure 6B.10.1 A**, List 2). Terms and pathways associated with this list included cytokine signalling, immune cell communication and the upregulation of some metabolic processes (**Figure 6B.10.1 C**). These terms were reflected by the presence of many cytokine and chemokine genes:

Il17f, Il17a, Il10, Il1a, Il3, Il24, Ccl3, Ccl4, Ccl5 (**Figure 6B.10.1 C**). *Il17a* and *Il10* were both found within this list and their mRNA expression profiles mirror the protein production profiles seen by ICS (**Figure 6B.10.1 D**). Of note, the levels of *Il17a* and *Il10* expression seen in the bulk populations are relatively low and seem to be masked by the IL-17- and IL-10- subpopulations, respectively.

As mentioned, the genes in this group were not all upregulated in all the cytokine producing subpopulations. The heat map reveals three clusters of genes that had obvious differences in their expression profiles (**Figure 6B.10.1 A**). The genes within these three lists are displayed in **Figure 6B.10.2**. Lists 1 and 3 were genes that were upregulated in both IL-17 producing subpopulations. List 2 was genes that were upregulated in both IL-10 producing subpopulations.

In List 1, the most highly upregulated gene in the IL-17+ IL-10- and IL-17+ IL-10+ subpopulations was *Il17a* (**Figure 6B.10.2**, List 1). Therefore the expression of genes in this cluster are likely to be regulated in parallel with *Il17a*. Additionally, *Nos2* is upregulated specifically in the IL-17 producing subpopulations, which is known to be produced by Th17 cells (Jianjun et al., 2013; Obermajer et al., 2013).

In List 2, one of the most highly upregulated genes in the IL-17- IL-10+ and IL-17+ IL-10+ subpopulations was *Il10* (**Figure 6B.10.2**, List 2). The most highly upregulated genes in the IL-17- IL-10+ and IL-17+ IL-10+ subpopulations were *1700094J05Rik* and *Dnahc11,* suggesting the expression of genes in this list are regulated in parallel with *Il10. Dnahc11* alongside *Klrc1, Klrc2* and *Klrc3* (**Figure 6B.10.2**, List 2 in bold) were all also found in Group 7 of the Th1 cell analysis in Chapter 5; these were genes most highly upregulated in the IFNγ+ IL-10+ subpopulations (**Figure 5B.12.1**). Therefore, these genes are associated with *Il10* expression in both Th1 cells and Th17 cells.

In List 3, many of the genes are highly upregulated in the IL-17+ IL-10- and IL-17+ IL-10+ subpopulations (**Figure 6B.10.2**, List 3). Overlap of this cluster with those genes found in Group 6 of the Th1 cell analysis in Chapter 5; these genes were upregulated in cytokine producing subpopulations except in the Th1 + IL-27 IFNγ+ IL-10+ subpopulation, suggesting an inverse correlation with IL-10 (**Figure 5B.11.1**), revealed many similar genes (**Figure 6B.10.2**, List 3 in bold): *Kcna5, Ebf1, Igf2as, Sdk1, Adamtsl1, Lhx9, Pbx1, Nodal, Megf10* and *Cpn2*. These genes were more associated

with *Ifng* expression than *Il10* expression in Th1 cells, and more associated with *Il17a* expression than *Il10* expression in Th17 cells, suggesting these genes are inversely correlated with *Il10* and could be potential repressors of *Il10* gene expression.

IL10 and IL17F played a central role in the interactions between these genes (**Figure 6B.10.3**). Interestingly, again, (as seen in **Figure 6B.7.3**) ESR1 is also a core node (**Figure 6B.10.3**).

6.4.5 Hierarchical clustering separates the Th17 + anti-IL-2 intracellular cytokine producing subpopulations into 2 groups based on their gene expression profiles

To further investigate the differential gene expression between the subpopulations within the Th17 + anti-IL-2 subset we applied statistical filtering to retain genes that were at least 2-fold up- or downregulated in at least one of the samples (repeat data pooled) vs. the baseline (median of all the samples). This resulted in a list of 106 genes that we then subjected to hierarchical clustering on entities and conditions (**Figure 6B.11**). The 106 gene set separated into two major branches; the bulk and IL-17+ IL-10- clustered separately from the IL-17- IL-10- subpopulation. There were two clear groups of genes with different transcriptional profiles within this 106 set of genes:

- Group 1: 58 genes decreased in the IL-17+ IL-10- subpopulation of the Th17 cells cultured in the absence of IL-2.
- Group 2: 48 genes increased in the IL-17+ IL-10- subpopulation of the Th17 cells cultured in the absence of IL-2.

6.4.5.1 Genes that are decreased in the IL-17+ IL-10- subpopulation of the Th17 cells cultured in the absence of IL-2

Group 1 consisted of 58 genes (**Figure 6B.12 A**) with decreased expression in the IL-17+ IL-10- subpopulation of the Th17 + anti-IL-2 cells (**Figure 6B.12 A&B**). Pathways and terms associated with this group included cellular responses to mechanical stimulus, PKA signalling and Th cell differentiation (**Figure 6B.12 C**). Genes included the

suppressor of cytokine signalling gene *Socs3,* the MCHII family member *H2-Ob, Il17c, Foxp3* and *Smad6* (**Figure 6B.12 C**). SOCS3, which is known to be involved in the downregulation of IL-17 production and Th17 development, was dramatically downregulated in the IL-17+ IL-10- subpopulation compared to the IL-17- IL-10- subpopulation of the Th17 + anti-IL-2 subset (**Figure 6B.12 D**). Furthermore, *Foxp3*, which suppresses Th17 development and promotes Tregs, was downregulated in the IL-17+ IL-10- subpopulation (**Figure 6B.12 D**), though the actual read numbers of *Foxp3* were very low and the inter-repeat variability resulted in large error bars. Therefore this decrease may not be physiologically relevant. There were few interactions between these genes (**Figure 6B.12 E**).

6.4.5.2 Genes that are increased in the IL-17+ IL-10- subpopulation of the Th17 cells cultured in the absence of IL-2

Group 2 consisted of 48 genes (**Figure 6B.13.1 A**) with increased expression in the IL-17+ IL-10- subpopulation of the Th17 + anti-IL-2 cells (**Figure 6B.13.1 A&B**). Pathways associated with these genes included immune cell communication, antigen presentation and iNOS signalling (**Figure 6B.13.1 C**). Genes in this list included *Batf3, Tgfbi* that encodes TGFβ induced protein*, Nos2* that encodes NOS2 / iNOS, and *Ifng* (**Figure 6B.13.1 C**). The read number of these genes was relatively low; nevertheless *Nos2, Ifng* and *Tgfbi* were all upregulated in the IL-17+ IL-10- subpopulation compared to the IL-17- IL-10- subpopulation (**Figure 6B.13.1 D**). The expression of *Nos2* was highest in the bulk and intermediate in the IL-17+ IL-10- subpopulation. The expression of *Ifng* was lower in the bulk Th17 + anti-IL-2 subset compared to the IL-17+ IL-10- subpopulation. The expression of *Tgfbi* in the bulk was about mid-way between the IL-17+ IL-10- and IL-17- IL-10- subpopulations (**Figure 6B.13.1 D**). Network analysis revealed IFNγ as a central node in the interactions of these genes, and there were indirect interactions between IFNγ and BATF3, and NOS2 (**Figure 6B.13.2**).

6.5 Discussion

IL-10 production by Th17 cells is variable and transient, and the mechanisms that are involved in driving robust IL-10 from Th17 cells are unknown. IL-2 is thought to be a Treg promoting cytokine (Davidson et al., 2007; Fontenot et al., 2005; Yamane and Paul, 2012) that suppresses Th17 cell differentiation and IL-17 production (Laurence et al., 2007). After investigating many factors that may have been involved in the upregulation of IL-10 production in Th17 cells we have found that IL-2 levels are crucial in driving two distinct forms of Th17 cell; addition of IL-2 results in increased IL-10, albeit alongside decreased IL-17, and blockade of IL-2 results in the disappearance of IL-10 alongside increased IL-17. Therefore, we wanted to characterise the transcriptional changes IL-2 drives in Th17 cells, and also to determine the effects of IL-2, and the process of cytokine production, had on these different intracellular cytokine producing subpopulations. Consequently we carried out RNA-Seq analysis on Th17 cells cultured with IL-2 or anti-IL-2, and on the intracellular cytokine producing subpopulations within these two subsets.

6.5.1 IL-2 drives increased IL-10 production, and decreased IL-17 production, from Th17 cells

In the absence of IL-2, achieved by the addition of anti-IL-2 antibody, Th17 cells produced substantial amounts of IL-17 and very little IL-10. In the presence of IL-2 Th17 cells produced significant amounts of IL-10, and a reduced amount of IL-17. However, these IL-10 producing Th17 cells did not have detectable amounts of FoxP3. The expression of *Il17a* and *Il10* mRNA matched that of IL-17 and IL-10 protein production, in the bulk Th17 + IL-2 and Th17 + anti-IL-2 subsets and in the different subpopulations. This suggests that IL-2 can drive IL-10 expression in Th17 cells in a FoxP3 independent and possibly STAT5 dependent manner. There is little mention in the literature of a role for IL-2 in driving IL-10 production by Th17 cells; however, this finding may explain other observations that have solely been attributed to the role of IL-2 on Tregs. IL-2 deficient mice suffer from unlimited inflammatory bowel disease (IBD) (Sadlack et al., 1993), the loss of lymphocyte homeostasis and autoimmunity (Suzuki et al., 1995). Therefore, it was concluded that IL-2 must have paradoxical roles in not only promoting T cell differentiation, but also in maintaining a regulated immune

response. The important role for IL-2 in maintaining Tregs offered a possible solution – when IL-2 is removed, Treg homeostasis is lost and therefore immune regulation is lost. However, our data suggests that IL-2 may also have a direct role in maintaining Th17 regulation by promoting IL-10 production from these cells, which could then act in an autocrine manner to prevent Th17 driven pathology (Huber et al., 2011). This theory is supported by the important role Th17 cells play in autoimmunity (Miossec et al., 2009) and colitis (Elson et al., 2007; Lee et al., 2009b), and the absolute requirement for IL-10 in gut homeostasis (Kuhn et al., 1993). Therefore, IL-2 may not only drive Treg homeostasis but also IL-10 producing Th17 cells, which together could act to prevent immune dysregulation and gut pathology. However, this phenotype is likely to be highly dose dependent and greater concentrations of IL-2 may enable competition between STAT5 and STAT3, which would prevent Th17 differentiation. In this context, we have optimised a system in which we can consistently drive IL-10 production from Th17 cells without compromising the stability of their differentiation.

6.5.2 Culture in the presence or absence of IL-2 results in changes in the transcriptional profile of bulk populations of Th17 cells

Analysis of the transcriptional profiles of the bulk unseparated Th17 + IL-2 and Th17 + anti-IL-2 subsets, revealed that the cells within each subset were transcriptionally very different. Unlike what we saw with the analysis of the Th1 and Th1 + IL-27 cells in Chapter 5, analysis of the different intracellular cytokine producing subpopulations within the Th17 + IL-2 and Th17 + anti-IL-2 subsets revealed very similar results to the analysis of the bulk unseparated subsets. This may be explained by the IL-2 or anti-IL-2 conditions resulting in opposite transcriptional profiles in the Th17 cells. Therefore, the majority of the genes were revealed in the analysis of bulk unseparated Th17 + IL-2 and Th17 + anti-IL-2 subsets. Nevertheless some genes, including *Il10, Il17a, Socs3* and *Foxm1*, were revealed by separating the heterogeneous bulk Th17 + IL-2 and Th17 + anti-IL-2 subsets (**Discussion figure 6.1**).

In Th17 cells cultured in the presence of IL-2 or anti-IL-2 we see an increase in the expression of *Socs3* in the IL-17- IL-10- subpopulations compared to the cytokine producing subpopulations (**Discussion Figure 6.1**). However, in the absence of IL-2 this increase in the expression of *Socs3* is much more dramatic (**Discussion Figure 6.1**).

IL-2 did not affect the expression of *Socs3* in the cytokine producing subpopulations. Therefore, our data suggests that one of the major differences between the IL-17- IL-10- subpopulation and cytokine producing subpopulations is the expression of *Socs3*, and that IL-2 may downregulate *Socs3*. Suppressor of cytokine signalling (SOCS) proteins are associated with the suppression of cytokine production and there is much evidence that SOCS3 downregulates Th17 cell differentiation, and IL-17 and IL-6 production (reviewed in (Tamiya et al., 2011)). SOCS proteins are classical negative feedback regulators; cytokine ligation results in JAK-STAT signalling that induces SOCS transcription, which in turn blocks cytokine receptor signalling and JAK-STAT phosphorylation. SOCS3 has been shown to prevent phosphorylation of STAT3 (Li et al., 2012b) and in doing so inhibit downstream STAT3 signalling. One mechanism by which it has been proposed to do this is via the inhibition of IL-23 signalling, which in turn phosphorylates STAT3 to promote IL-17 production (Chen et al., 2006). Furthermore, SOCS3 can block JAK2's ability to phosphorylate substrates (Babon et al., 2012). In summary, the increased levels of SOCS3 in the IL-17- IL-10- subpopulations of Th17 + IL-2 and Th17 + anti-IL-2 cells may be a major factor in preventing cytokine production by these cells.

Our data shows that in the bulk, IL-17- IL-10- and IL-17+ IL-10- subpopulations, IL-2 results in an upregulation of *Foxm1* expression (**Discussion Figure 6.1**). However, the IL-10 producing subpopulations of the Th17 + IL-2 subset have dramatically reduced *Foxm1* expression. FoxM1 is a master regulator of cell cycle and has been shown to be important for the proliferation of early thymocytes and activated CD4+ T cells (Xue et al., 2010). IL-2 is known to promote Th cell proliferation (Yamane and Paul, 2012) and therefore the upregulation of *Foxm1* expression by IL-2 seen in our results is in keeping with the literature. Therefore our data suggests that IL-2 upregulates *Foxm1* expression to drive the proliferation of the Th17 cells, but this expression is subsequently downregulated in the IL-10 producing subpopulations.

6.5.2.1 *Maf* and *Prdm1* are upregulated in the presence of IL-2

c-Maf clearly required IL-2 for its expression since its expression was abrogated when anti-IL-2 was added to the cultures (**Discussion Figure 6.2**). In Th17 cells c-Maf, downstream of TGFβ signalling, can induce genes involved in repressing inflammation,

such as *Il10* (Ciofani et al., 2012). Furthermore, in Th17 cells c-Maf has been shown to bind the *Il10* promoter and transactivate it, and thus regulate IL-10 expression (Xu et al., 2009). Furthermore, c-Maf has also been associated with AhR signalling: the interaction between the two has been shown to drive *Il10* transcription (Apetoh et al., 2010). We have shown here that in Th17 cells cultured in the presence of IL-2, *Maf* expression and *Il10* expression are upregulated, as in the presence of anti-IL-2 their expression is abrogated. Therefore it is possible that this increase in c-Maf, resulting from IL-2 signalling, may be driving the upregulation of *Il10.*

Our data also suggested that Blimp-1 (*Prdm1*) required IL-2 for its expression, as its expression was decreased in Th17 cells cultured in the absence of IL-2 (**Discussion Figure 6.2 A**). The *Prdm1* gene encodes Blimp-1, which has been shown to positively regulate IL-10 expression in Th cells; conditional deletion of *Prdm1* in T cells leads to pathology and inflammation *in vivo* and decreased IL-10 production by CD4+ T cells (Martins et al., 2006). It has been shown that IL-12 can induce Blimp-1 regulation of IL-10 via STAT4 in Th1 cells (Neumann et al., 2014) and that IL-10 expression in IL-12 driven Th1 cells was dependent on Blimp-1. This IL-10 production was enhanced by Blimp-1 synergising with c-Maf (Neumann et al., 2014).

IL-2 has been shown to induce *Maf* expression in human bulk CD4+ T cells via STAT5, which directly binds to the *Maf* locus (Rani et al., 2011). Also in CD8+ T cells IL-2 has been shown to promote IL-10 production via a Blimp-1 dependent mechanism (Sun et al., 2011). Therefore, it is possible that in Th17 cells IL-2 promotes the expression of c-Maf and Blimp-1, which then cooperate to drive *Il10* expression; it may be via this mechanism that IL-2 drives IL-10 production in Th17 cells.

6.5.2.2 AhR signalling is upregulated in the presence of IL-2

Our data shows that in presence of IL-2 there is upregulation in the expression of molecules involved the repression of in AhR signalling, namely *Ahrr* and *Cyp1b1* (**Discussion Figure 6.2 B**). The AhR has important physiological functions in cellular development and immunity, however the effects of AhR on immune responses are multidimensional and variable. It can induce both immunosuppressive functions, and pathogenic T cell responses. *Ahrr* and *Cyp1b1* are often upregulated by AhR signalling

(Hao and Whitelaw, 2013) and upregulation of these genes by IL-2 within our data suggests that IL-2 induces AhR signalling. The ligation of AhR with its classical ligand TCDD leads to the suppression of EAE via the induction of Treg cell differentiation (Zhang et al., 2010). In a graft-versus-host model, TCDD has been shown to promote the development of Treg cells that produced significant amounts of IL-10 (Marshall et al., 2008). Therefore in certain scenarios AhR signalling is known to skew CD4+ T cells towards a Treg phenotype and to promote the production of IL-10 by Tregs in various ways. Our data suggests that can IL-2 promote AhR signalling, and that via this pathway IL-2 may be promoting the IL-10 producing phenotype of Th17 cells that we observe.

6.5.2.3 Culture of Th17 cells with IL-2 results in the increase in *Il10ra, Ifng* and *Nos2* expression

In our system, the addition of anti-IL-2 to Th17 cell cultures abolished the expression of the *Il10ra* (**Discussion Figure 6.2 C**). This increase in expression of the IL-10 receptor by IL-2 is likely to result in Th17 cells being more responsive to IL-10, which in turn has been shown to negatively regulate IL-17 production by Th17 cells (Huber et al., 2011).

We show that low levels of *Ifng* expression were induced by IL-2 signalling (**Discussion Figure 6.2 C**), supporting the idea that Th17 cells can take on a more 'regulatory' Th1 like phenotype, expressing IL-10 and trace amounts of IFNγ (Gagliani et al., 2015). In this paper they suggest that Th17 cells can transdifferentiate into IL-10+ (Tr1) regulatory cells (Gagliani et al., 2015), and we propose a mechanism by which this might occur, via IL-2 signalling. *In vitro* and *in vivo* analysis of IL-2 receptor deficient Th17 cells could be used to address this and to further investigate the role of IL-2 in driving the conversion of Th17 cells into IL-10+ regulatory cells. However, owing to the importance of IL-2 signalling in multiple pathways of Th cell proliferation and differentiation, conditional deletion of the IL-2R in a temporally controlled manner would likely be necessary.

In our samples we see that IL-2 leads to an upregulation of *Nos2* expression in Th17 cells (**Discussion Figure 6.2 C**). Furthermore, we see an increase in NOS2 expression in the IL-17+ subpopulations of both the Th17 + IL-2 and Th17 + anti-IL-2 subsets compared to the IL-17- IL-10- subpopulations (**Discussion Figure 6.2 C**), although the actual amount of NOS2 mRNA we see in our samples are very low. It has been shown that human and mouse Th17 cells can produce NOS2 (Jianjun et al., 2013; Obermajer et al., 2013), though the functional role of this enzyme is still unresolved, with both positive and negative effects on Th17 cell cytokine production having been proposed. In mouse Th17 cells NOS2 has been shown to hinder Th17 cell differentiation via the inactivation of RORγt (Jianjun et al., 2013). While in human Th17 cells endogenous NOS2 and NO-induced cGMP signalling have been shown to maintain Th17 cell *Il17* and *Il23r* expression (Obermajer et al., 2013). Therefore, we cannot know the actions of the endogenous expression of NOS2 seen in our Th17 cells. However, as it is more highly expressed in the IL-17+ subpopulations our data supports the theory that NOS2 is promoting *Il17* expression as suggested by Obermajer et al.

6.5.2.4 IL-2 downregulates the expression of molecules involved in retinoic acid metabolism

We show that the expression of *Cyp26a1* and *Cyp26b1*, two proteins fundamental to the degradation of retinoic acid, were downregulated in the presence of IL-2 since they were increased by the presence of anti-IL-2 in the cultures (**Discussion Figure 6.3 A**). Retinoic acid (RA) is derived from vitamin A and ligates nuclear RA receptors (RARs) and retinoid X receptors (RXRs). The distribution and levels of RA are tightly controlled by synthesis and degradation by specific cytochrome P450s (Cyp26) (Rhinn and Dolle, 2012). In CD4+ T cells Cyp26b1 has been identified as the primary negative regulator of RA, and Cyp26b1 deficiency is associated with reduced intestinal inflammation and the reduced development of IFNγ producing 'pathological' Th17 cells (Chenery et al., 2013). In fact RA alters the balance between Th17 cells and Tregs, in favour of Tregs. RA enhances the expression and phosphorylation of SMAD3, which enables enhanced TGFβ signalling, resulting in increased expression of FoxP3 (Xiao et al., 2008). To further promote Treg development and prevent Th17s, RA also inhibits the expression of *Il6ra* and *Il23r* (Xiao et al., 2008). IL-23 signalling is known to reduce

IL-10 production by Th17 cells and promote 'pathogenic' Th17 cells (Ghoreschi et al., 2010; McGeachy et al., 2007). Therefore, this data suggests that by repressing the metabolism of RA in Th17 cells, IL-2 promotes IL-10 production and the differentiation of Th17 cells in the absence of FoxP3.

6.5.2.5 The expression of genes involved in ERK / AP-1 signalling is altered by IL-2

Il2 expression was dramatically increased in Th17 cells cultured in the absence of IL-2, suggesting that the lack of IL-2 drives *Il2* expression, and that the presence of IL-2 represses *Il2* expression (**Discussion Figure 6.3 B**). IL-2 negatively regulates Th17 cell differentiation (Laurence et al., 2007), but Th17 cells are likely to need low levels of IL-2 early in differentiation to promote proliferation. These data suggest that IL-2 signalling results in a negative feedback loop to maintain low levels *Il2* expression, possibly to allow Th17 differentiation.

In the list of genes that were downregulated by IL-2, and thus increased by the addition anti-IL-2, there were various factors that have been associated with AP-1 signalling, namely *Twist1, Egr1* and *Egr3* and *Fos*/*Fosb* (**Discussion Figure 6.3 B**). AP-1 is downstream of the MAP kinase ERK, which is activated via RAS downstream of the TCR. AP-1 is a heterodimeric transcription factor composed of dimers of the FOS, JUN, JUN dimerising protein (JDP) and activating transcription factor (ATF) family members (Karin et al., 1997). AP-1 signalling is highly complex and incompletely understood; there are many different types of AP-1 dimers, all with different functional roles. However, many of the ERK and AP-1 family members present in our gene list have been associated in the regulation of IL-2 (Macian et al., 2002; Whitehurst and Geppert, 1996).

AP-1 has been shown to interact with NFAT, which results in the integration of Calcium and Ras signals and the expression of genes important for T cell activation including IL-2 (Macian et al., 2002). There are two AP-1 sites within the *Il2* promoter at which various combinations of Fos and Jun are thought to bind (Jain et al., 1992). In CD4+ T cell lines c-Jun, JunD, JunB and FosB are thought to interact with Oct proteins to increase *Il2* promoter activity (Foletta et al., 1998). Furthermore, Egr3 blocks BATF

(an AP-1 inhibitor) and in doing so promotes AP-1 activation and IL-2 production in CD4+ T cells (Li et al., 2012b). In the absence of IL-2, Th17 cells increase the expression of these ERK / AP-1 genes. Therefore it is possible that this is a feedback mechanism; in the presence of IL-2 the Th17 cells downregulate pathways that promote *Il2* expression, and thus maintain Th17 differentiation, which is downregulated by IL-2. However, further investigation would be needed to address the precise function of these transcription factors in these circumstances.

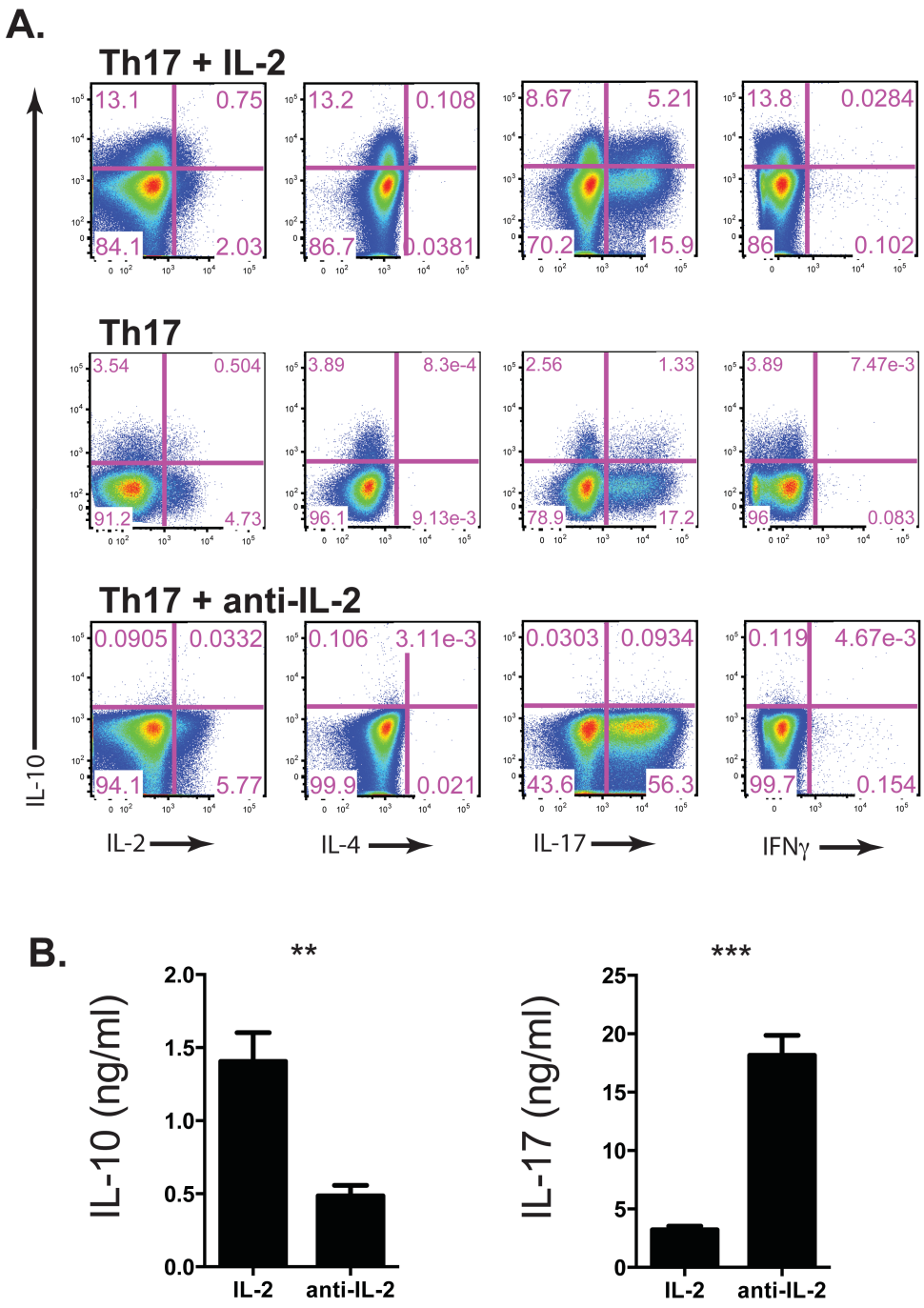


Figure 6A.1 IL-2 is essential for IL-10 production, but inhibits IL-17 production, by Th17 cells

C57BL/6 naive CD4+CD62L+CD44loCD25- cells were driven with plate-bound anti-CD3 and soluble anti-CD28, and polarised to Th17 cells with IL-6, TGFβ, anti-IL-12, anti-IFNγ and anti-IL-4 in the presence or absence of IL-2 (Th17 + IL-2) or anti-IL-2 (Th17 + anti-IL-2), or neither (Th17). **A.** Plots of flow cytometric analysis of intracellular cytokine staining. Numbers show percentage of live CD4+ cells. Gating strategy described in Materials and Methods. **B.** Graphs represent cytokines in supernatants determined by ELISA. Cells restimulated as described in Materials and Methods, assessed after 3 days of polarization *in vitro*. Graphs show means ± SD of triplicates, **p < 0.01, ***p < 0.001, ****p<0.0001 determined by students T-test. Representative of three experiments.

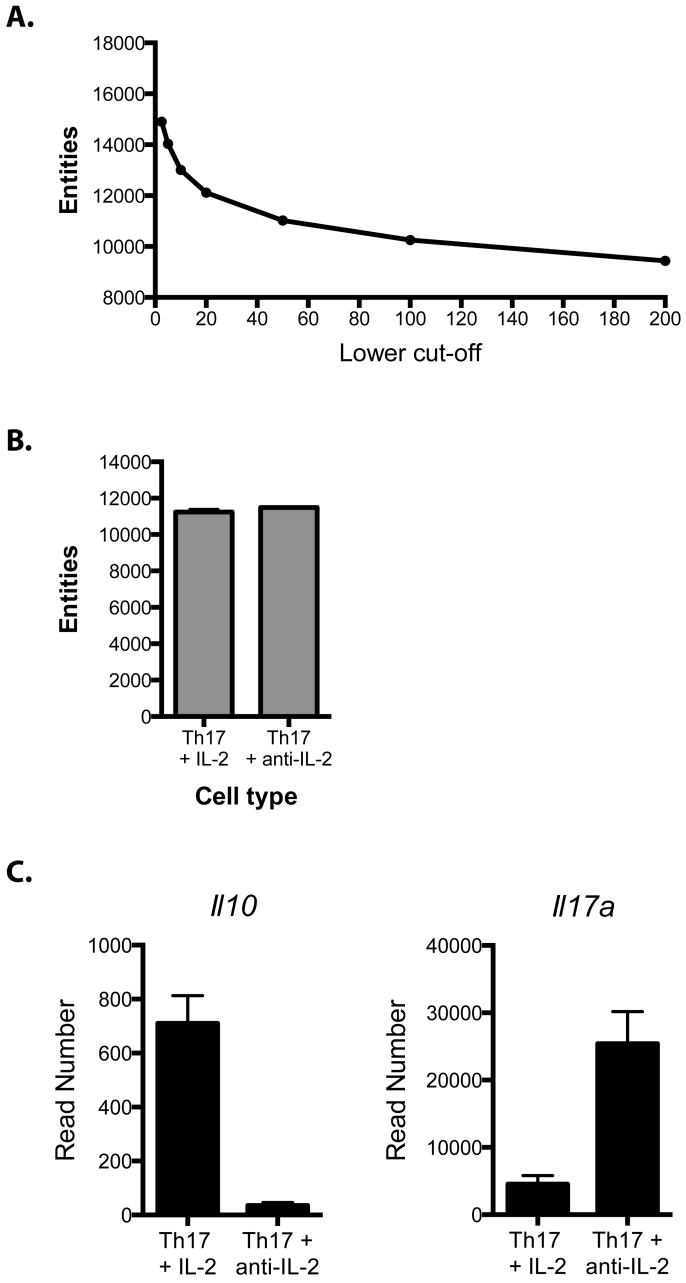


Figure 6A.2 Determining the optimal lower cut-off of reads for expression threshold of bulk Th17 + IL-2 and Th17 + anti-IL-2 RNA-Seq samples

mRNA extracted and prepared for RNA-Seq as described in Materials and Methods. Using Strand NGS software reads were aligned to the transcriptome & Genome (mm10, RefSeq annotation, 95% identity, max 5% gaps, 1 read only if duplicate) and normalisation with DeSeq and no Baseline. Upper cut-off 253033.02 (where at least 1 out of 6 samples have values within cut-off). **A.** The number of entities passing expression thresholds with indicated lower cut-offs. All bulk Th17 + IL-2 and Th17 + anti-IL-2 samples were pooled for this test. **B.** Using an expression threshold with a lower cut-off of 20 reads, the number of entities in each repeat within the bulk Th17 + IL-2 and Th17 + anti-IL-2 subsets was determined. **C.** Graphs of *Il10* and *Il17a* read number from the RNA-Seq data.

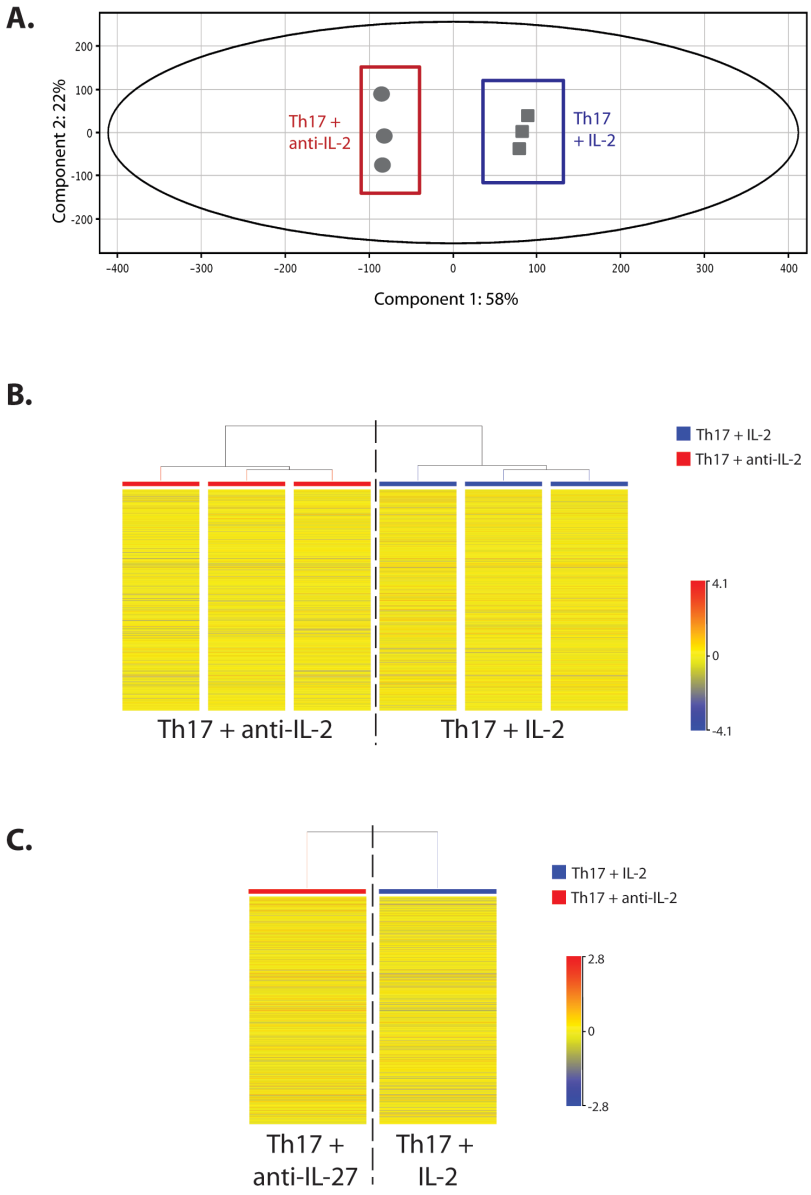
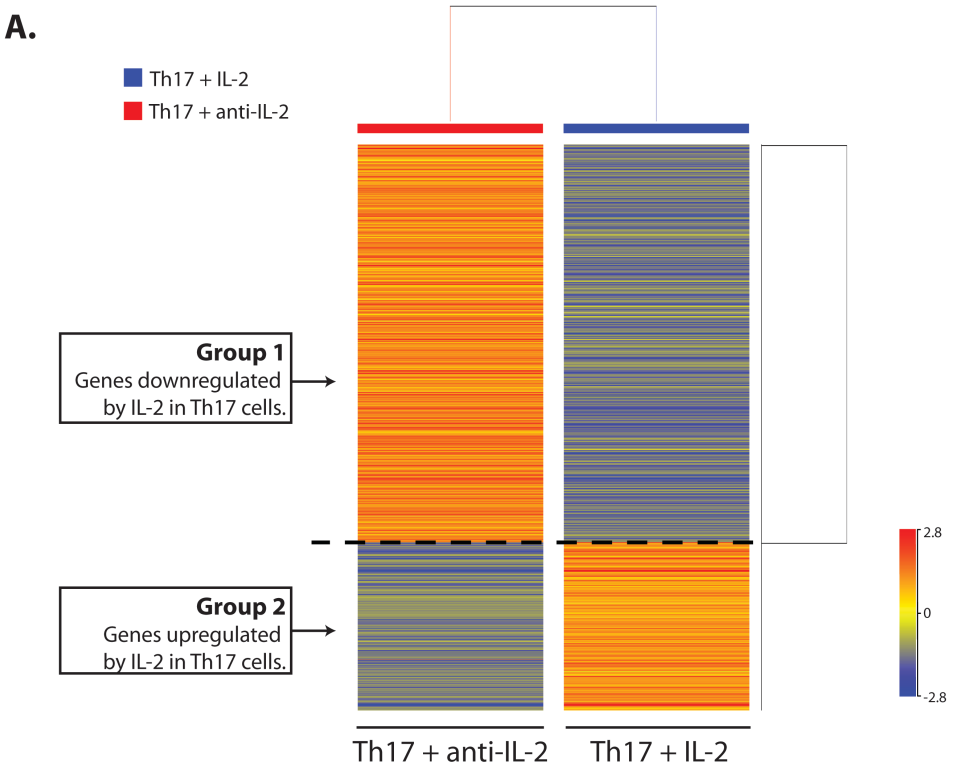


Figure 6A.3 PCA and cluster analysis demonstrates that bulk Th17 + IL-2 and Th17 + anti-IL-2 cells have different gene expression profiles

mRNA extracted and prepared for RNA-Seq as described in the Materials and Methods. **A.** PCA plots of each repeat of the bulk Th17 + IL-2 and Th17 + anti-IL-2 samples analysed by RNA-Seq. **B.** Unsupervised hierarchical clustering on conditions, of all individual repeats within the bulk Th17 + IL-2 and Th17 + anti-IL-2 subsets. **C.** Unsupervised hierarchical clustering on conditions, the bulk Th17 + IL-2 and Th17 + anti-IL-2 subsets (repeat data pooled). Unsupervised hierarchical clustering was carried out on the normalised intensity values with a Pearson's centred similarity measure and an Average linkage rule. Colour ranges indicate normalised intensity. The dendrogram represents the relatedness of the samples.



B. Group 1

606 genes downregulated by IL-2 in Th17 cells.

Associated GO terms	Associated IPA Pathways	Genes Included
Retinoic acid metabolic process, Cell adhesion, Regulation of phosphate metabolic process	FXR/RXR activation, P2Y purigenic receptor signalling, MIF regualtion of innate immunity (JNK/AP1 signalling)	*Cyp26a1, Cyp26b1, Aldh8a1, Il2, Il23a, Il17a, Fos, Fosb, Twist1, Egr1, Egr3*

C. Group 2

256 genes upregulated by IL-2 in Th17 cells.

Associated GO terms	Associated IPA Pathways	Genes Included
Cytokine receptor activity, Immune system effector process, Cell migration, Inflammatory response, Immune response	CTLA4 signalling, IL-12 signalling, T helper cell differentiation, CD28 signalling in T helper cells, IL-10 signalling	*Il10ra, Il12rb1, Il12rb2, Cxcr1, Ccr2, Ccr4, Ccr5, Il10, Il24, Maf, Prdm1, Nos2, Cd80, Cd86*

Figure 6A.4.1 There are dramatic transcriptional differences between the bulk Th17 + IL-2 and Th17 + anti-IL-2 subsets

mRNA extracted and prepared for RNA-Seq as described in the Materials and Methods. Data from the three biological repeats were pooled. Differentially regulated genes were obtained by taking those that were at least 3-fold up- or downregulated in at least 1 of the 2 samples vs. the baseline (median of all the samples). **A.** This left 861 genes that were subjected to hierarchical clustering on entities and conditions with a Pearson's centred similarity measure and an Average linkage rule. Colour ranges indicate normalised intensity. The dendrogram represents the relatedness of the samples. Genes were separated into 2 groups based on the hierarchical dendrogram. **B.** Group 1 GO terms ($p<0.01$) and IPA pathways ($p<0.01$) with significant overlap, and examples of genes within the group. **C.** Group 2 GO terms ($p<0.01$) and IPA pathways ($p<0.01$) with significant overlap, and examples of genes within the group.

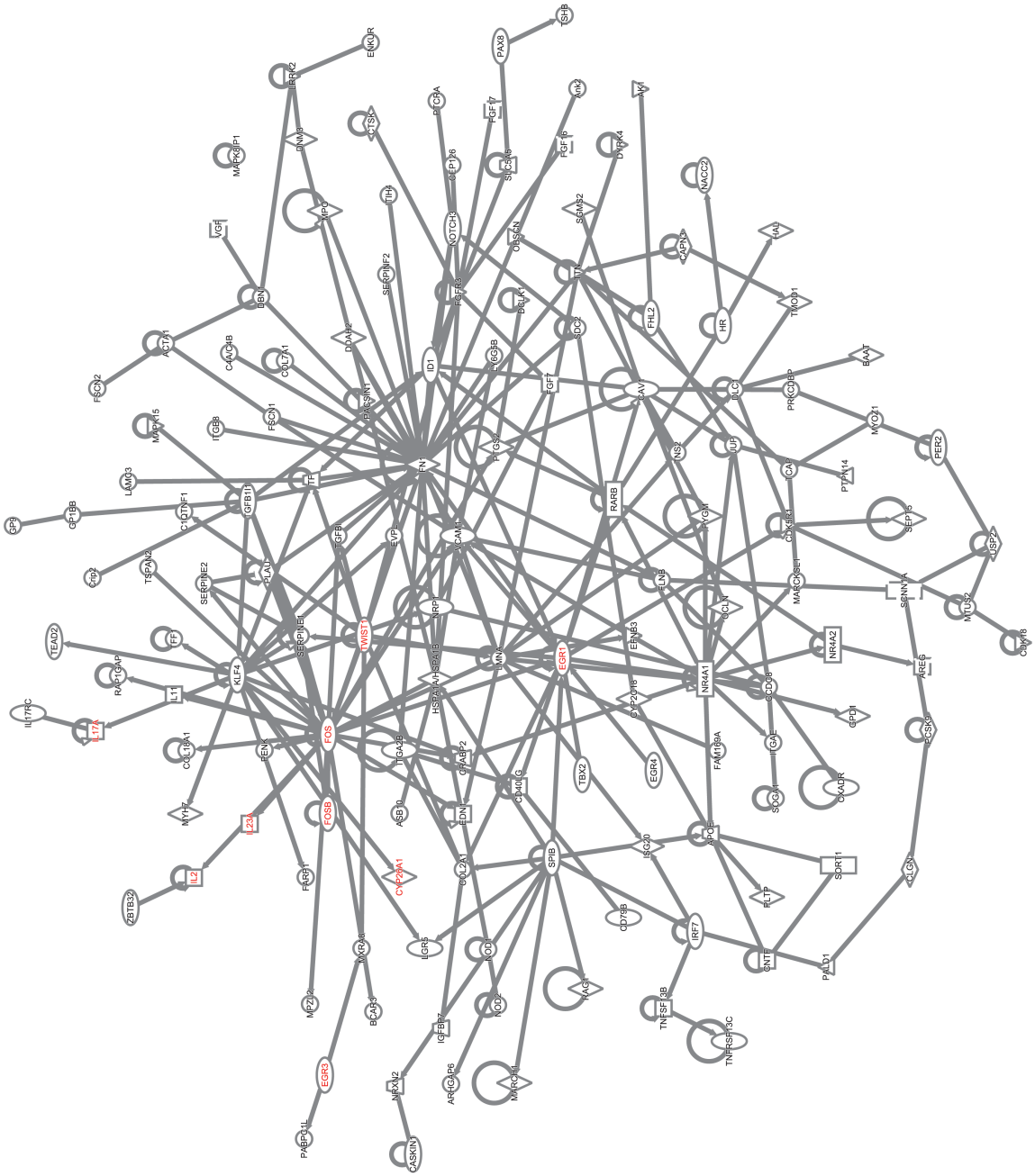


Figure 6A.4.2 Group 1: Network analysis of 606 genes downregulated by IL-2 in Th17 cells

IPA network analysis of direct interactions between genes within group 1 from Figure 6A.4.1. Genes in red are those discussed.

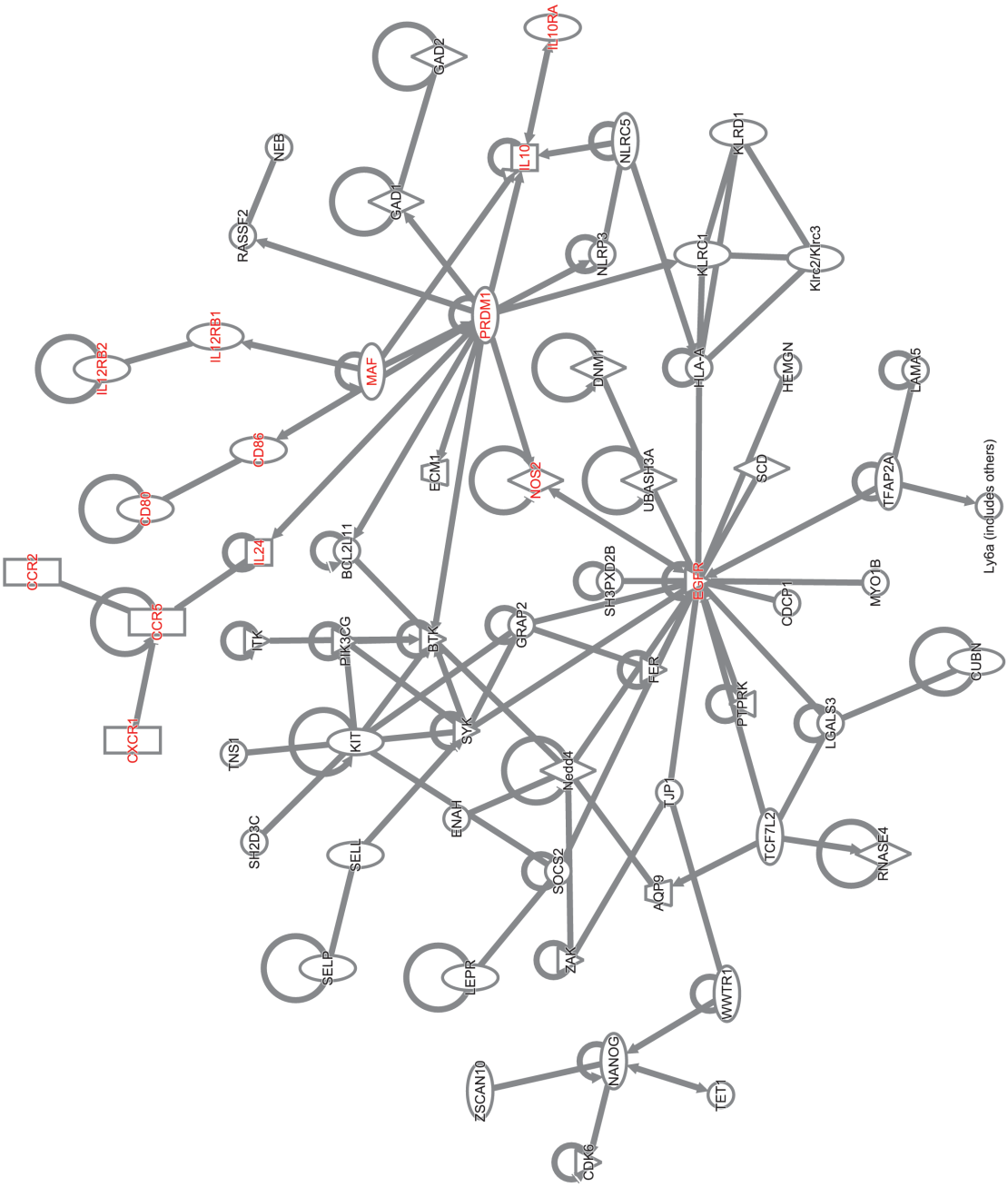


Figure 6A.4.3 Group 2: Network analysis of 256 genes upregulated by IL-2 in Th17 cells

IPA network analysis of direct interactions between genes within group 2 from Figure 6A.4.1. Genes in red are those discussed.

Table 6A.4.1 Group 1: List of 606 genes downregulated by IL-2 in Th17 cells

0610009L18Rik	*Adam1a*	*Bmp1*	*Clgn*
1700001J03Rik	*Adh4*	*Bnipl*	*Cnksr1*
1700001P01Rik	*Adig*	*Bok*	*Cntf*
1700003E16Rik	*Adra2a*	*Bpifc*	*Cntnap1*
1700007K09Rik	*Adra2b*	*Bsn*	*Col18a1*
1700029J07Rik	*AI414108*	*Bzrap1*	*Col20a1*
1700092M07Rik	*AI836003*	*C1qtnf1*	*Col2a1*
1700094J05Rik	*Aif1l*	*C1qtnf5*	*Col4a4*
1700102P08Rik	*Ak1*	*C4a*	*Col7a1*
1810010D01Rik	*AK129341*	*Cabp5*	*Col9a3*
1810019D21Rik	*Ak7*	*Cacna1g*	*Cplx1*
1810024B03Rik	*Aldh8a1*	*Cacna1s*	*Cplx3*
1810043G02Rik	*Alpk3*	*Camkv*	*Cpne9*
1810046K07Rik	*Amh*	*Capn3*	*Cpxm1*
2200002D01Rik	*Amhr2*	*Car15*	*Crabp2*
2310002J15Rik	*Ampd1*	*Car7*	*Crip2*
2310014L17Rik	*Ank2*	*Casc1*	*Crtam*
4930415F15Rik	*Ankrd13d*	*Caskin1*	*Ctrl*
4930428O21Rik	*Ankrd53*	*Cav1*	*Ctsk*
4930502E09Rik	*Ankrd7*	*Ccdc151*	*Ctxn1*
4930506M07Rik	*Aoc3*	*Ccdc169*	*Cuzd1*
4930538K18Rik	*Aph1c*	*Ccdc42b*	*Cxadr*
4930548H24Rik	*Apoe*	*Ccdc74a*	*Cxxc5*
4930550C14Rik	*Aqp7*	*Ccdc8*	*Cybrd1*
4930562C15Rik	*Areg*	*Ccin*	*Cyp26a1*
4930590J08Rik	*Arhgap6*	*Ccl25*	*Cyp26b1*
4931429I11Rik	*Arl9*	*Ccr8*	*Cyp2c55*
4933405L10Rik	*Armc9*	*Cd164l2*	*Cyp2t4*
4933408B17Rik	*Arntl2*	*Cd24a*	*Cyr61*
4933413G19Rik	*Asb10*	*Cd40lg*	*D6Ertd527e*
4933413J09Rik	*Ascl4*	*Cd5l*	*Daam2*
4933422A05Rik	*Asgr1*	*Cd72*	*Dbn1*
4933432I09Rik	*Atoh8*	*Cd79b*	*Dclk1*
6230400D17Rik	*Atp1a2*	*Cd96*	*Ddah2*
6330403K07Rik	*Atp6v0d2*	*Cd97*	*Ddn*
9230110C19Rik	*Atp6v0e2*	*Cdk18*	*Depdc7*
9830001H06Rik	*Avil*	*Cdk5r1*	*Dgki*
A330009N23Rik	*B230216G23Rik*	*Cel*	*Dhrs9*
A3galt2	*B3galt2*	*Celf5*	*Diras1*
AA986860	*B3gnt4*	*Celsr3*	*Dixdc1*
Abcg4	*Baat*	*Chadl*	*Dkkl1*
Abi3bp	*BC089491*	*Chd5*	*Dlc1*
Acacb	*Bcar3*	*Chrm1*	*Dlgap1*
Acta1	*Bdh2*	*Cib2*	*Dll3*
Actl7a	*Bdkrb1*	*Cldn15*	*Dnahc10*
Actl7b	*Best2*	*Clec4a1*	*Dnahc11*

Dnm3
Dok3
Dram1
Dusp18
Dyrk4
E530011L22Rik
Ecm2
Edn1
Efcab4a
Efcab5
Efhd1
Efna2
Efnb3
Egr1
Egr3
Egr4
Elavl3
Enkur
Epb4.1l5
Epb4.9
Epha8
Ephx3
Ermap
Esam
Espnl
Esrp2
Etnk2
Evpl
Fads3
Fam101a
Fam131a
Fam167a
Fam169a
Fam194a
Fam81a
Fam83e
Farp1
Fbxo36
Fermt2
Fes
Fgf16
Fgf17
Fgf7
Fgfr3
Fhl2
Flnb
Fn1
Fndc4
Fndc8
Fos
Fosb
Foxo6
Fscn1
Fscn2
Gabrr2
Gal3st1
Galr2
Gas2l1
Gbx1
Gch1
Gdf1
Gdf9
Gfra1
Ghrl
Gja10
Gjc2
Gldc
Glis1
Gm10390
Gm10767
Gm1123
Gm11517
Gm128
Gm16197
Gm16853
Gm4980
Gm514
Gm5176
Gm5544
Gm5741
Gm766
Gm996
Gnb3
Gng3
Gng4
Gnmt
Gp1bb
Gp9
Gpd1
Gpr152
Gpr153
Gpr3
Gpr35
Gpr62
Greb1l
Grin2c
Grin3a
Guca1b
Gucy1a3
Gzmm
Hal
Hapln4
Hbegf
Hddc3
Hecw2
Hhatl
Hhipl2
Hist1h2bc
Hpn
Hr
Hsd17b13
Hspa1a
Htra4
Id1
Idi2
Igfbp7
Igj
Il11
Il17a
Il17rb
Il17rc
Il17re
Il2
Il23a
Irf7
Isg20
Itga2b
Itgae
Itgb8
Itih4
Jam2
Jam3
Jph3
Jsrp1
Jup
Kcnc1
Kcnh5
Kcnj14
Kcnk10
Kcnk4
Kcnrg
Kif17
Kif9
Klf4
Klhl30
L3mbtl1
Lamc3
Lct
Lctl
Lgr5
Lin7b
Lipg
Lman1l
Lmna
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Lrrd1
Lrrk2
Lrrn4cl
Ly6g5b
Map3k9
Mapk15
Mapk8ip1
Mar1
Marcksl1
Mc1r
Mc5r
Mcam
Mfrp
Mfsd2b
Mlf1
Mn1
Morn3
Mospd4
Mpo
Mpzl2
Mslnl
Mst1r
Mtus2
Muc1
Mxra8
Myh3
Myh7
Myo15
Myo1e
Myoz1
Naaladl1
Nacc2
Nav1
Ncald
Ngb
Nhlh1

Nkg7
Nod1
Nod2
Notch3
Nox1
Noxo1
Npas1
Npc1l1
Npm2
Nr4a1
Nr4a2
Nrgn
Nrp1
Nrxn2
Nudt17
Nup62cl
Nxf7
Oaf
Oas1b
Obscn
Ocln
Olfm2
Olfr536
Olfr56
Olfr60
Olfr920
Opn1sw
Opn3
Osr2
Otoa
Otop1
Ovgp1
P2rx2
Pabpc1l
Pacsin1
Pald1
Palld
Pax8
Pcsk1
Pcsk6
Pcsk9
Pdcd1lg2
Pdzd3
Pdzrn3
Penk
Per2
Pianp
Pip5kl1
Pla2g4b
Pla2g4c
Pla2g4f
Plau
Plcd3
Plcd4
Plce1
Pld4
Plekhg4
Plekhg6
Pllp
Plscr2
Pltp
Plxnc1
Pnpla1
Pon3
Ppfia4
Ppil6
Ppp1r1a
Ppp1r32
Prkcdbp
Prnd
Proca1
Proz
Prr7
Prrt1
Prss46
Psors1c2
Ptch2
Ptcra
Ptgs2
Ptpn14
Pvrl1
Pygm
Pyroxd2
Qrich2
Rab13
Rag1
Rap1gap
Rarb
Rasd1
Rasd2
Rbp4
Rbpms2
Rdh1
Rdh9
Rem2
Rep15
Rgs8
Rho
Rhou
Rnf150
Rnf223
Rnf39
Ropn1l
Rsc1a1
Rsph9
Scd4
Scn4a
Scnn1a
Sdc2
Sec16b
Sema4c
Sep5
Serinc4
Serpine1
Serpine2
Serpinf2
Sez6l2
Sgms2
Sh2d4b
Sh2d5
Sh3bgrl2
Shd
Shisa4
Slc16a8
Slc17a6
Slc17a8
Slc22a13
Slc23a3
Slc27a5
Slc34a3
Slc39a10
Slc4a5
Slc5a2
Slc5a5
Slc6a17
Slc8a2
Slit1
Smagp
Smpd3
Snta1
Sort1
Spem1
Spib
Spin4
Spns2
Ssc5d
Sspo
Stk30
Sult1a1
Susd2
Syngr4
Syt7
Tagln3
Tas1r1
Tbr1
Tbx2
Tcap
Tcfl5
Tcp11
Tead2
Tecta
Tekt2
Tenc1
Tff1
Tgfb1i1
Tgfbi
Thsd1
Timd2
Tktl1
Tlr13
Tm6sf2
Tmem151b
Tmem176a
Tmem176b
Tmem231
Tmem236
Tmem25
Tmem30c
Tmem44
Tmem51
Tmem88
Tmod1
Tmprss3
Tmprss9
Tnfrsf13c
Tnfsf13b
Tnn
Tnnt3
Tppp
Tppp3
Trf
Trim10

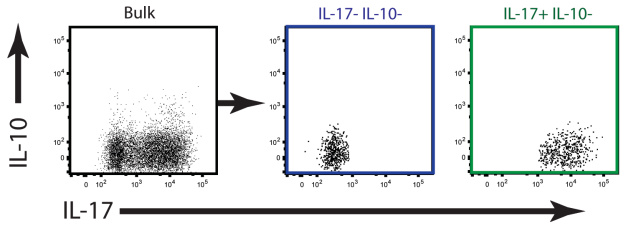
Trim15	*Ttc36*	*Vil1*	*Zbtb32*
Trim72	*Ttn*	*Vipr2*	*Zfp651*
Tshb	*Tub*	*Vmn1r32*	*Zfp811*
Tspan2	*Twist1*	*Vmn2r97*	*Zfp819*
Tssk1	*Ucn2*	*Vwa5b2*	*Zfp941*
Tssk2	*Unc13b*	*Vwa7*	*Zglp1*
Tssk3	*Usp2*	*Wasf1*	*Zmynd10*
Ttbk1	*Utf1*	*Wnk4*	*Zmynd15*
Ttc21a	*Vcam1*	*Xcl1*	
Ttc25	*Vgf*	*Xkrx*	

Table 6A.4.2 Group 2: List of 256 genes upregulated by IL-2 in Th17 cells

1110054M08Rik	*C1qtnf6*	*Fgf2*	*Il24*
1500017E21Rik	*Cass4*	*Fgl2*	*Ildr1*
1700058G18Rik	*Ccr2*	*Folr4*	*Inha*
1700066B19Rik	*Ccr4*	*Foxf2*	*Itga7*
1700097N02Rik	*Ccr5*	*Gad1*	*Itih5*
1700123M08Rik	*Cd200r1*	*Gad2*	*Itk*
1810034E14Rik	*Cd200r4*	*Gap43*	*Kbtbd13*
2010005H15Rik	*Cd226*	*Gbp8*	*Kcnj8*
2210408F21Rik	*Cd80*	*Gimap4*	*Kit*
2610528A11Rik	*Cd86*	*Gimap7*	*Klrc1*
2900011O08Rik	*Cd93*	*Gipr*	*Klrc2*
4930452B06Rik	*Cdcp1*	*Gja5*	*Klrc3*
4930539E08Rik	*Cdk6*	*Gli3*	*Klrd1*
4932438H23Rik	*Cnnm1*	*Gliprl*	*Klre1*
5031414D18Rik	*Cox4i2*	*Gm10390*	*Klri2*
5033411D12Rik	*Cpne6*	*Gm10638*	*Kremen2*
5830416P10Rik	*Ctla2a*	*Gm10865*	*Lair1*
6430571L13Rik	*Ctla2b*	*Gm11346*	*Lama5*
9130015A21Rik	*Cubn*	*Gm12185*	*Lbp*
9930111J21Rik1	*Cwh43*	*Gm13546*	*Lepr*
a	*Cxcr1*	*Gm14005*	*Lgals3*
A630023P12Rik	*Cyp1a1*	*Gm16548*	*Lhfpl2*
A630066F11Rik	*Cyp1b1*	*Gm1966*	*Lhx6*
Abcb9	*Cysltr1*	*Gm20098*	*Lrfn3*
Abcc9	*D830046C22Rik*	*Gm4827*	*Lrmp*
Abcg3	*Disc1*	*Gm5431*	*Lrrc32*
Acsl6	*Dleu7*	*Gm5483*	*Ltb4r1*
Adamtsl4	*Dnahc8*	*Gm6455*	*Ly6c1*
Agphd1	*Dnm1*	*Gphb5*	*Lzts1*
Ahrr	*Dntt*	*Gpr114*	*Maf*
Alpk2	*E130218I03Rik*	*Gpr146*	*Map2k6*
Ampd3	*Ecm1*	*Gpr15*	*Mcf2l*
Angptl6	*Egfr*	*Gpr55*	*Micall2*
Ankrd34b	*Enah*	*Grap2*	*Myo16*
Anxa3	*Enpp1*	*Grm6*	*Myo1b*
Aqp9	*Entpd1*	*Gucy2e*	*Myof*
Arap3	*Exd1*	*H2-Q1*	*Naip2*
Arhgap19	*F2rl2*	*H60b*	*Nanog*
Arhgap25	*Faim3*	*Hemgn*	*Nap1l5*
Arhgef39	*Fam110c*	*Hmx2*	*Neb*
Asb2	*Fam20a*	*Hrh2*	*Nedd4*
B230120H23Rik	*Fam26f*	*Hsf2bp*	*Neurl3*
B430306N03Rik	*Fam71b*	*Il10*	*Nhs*
Bcl2l11	*Fanci*	*Il10ra*	*Nlrc5*
Btbd8	*Fert2*	*Il12rb1*	*Nlrp3*
Btk	*Ffar2*	*Il12rb2*	*Nos2*

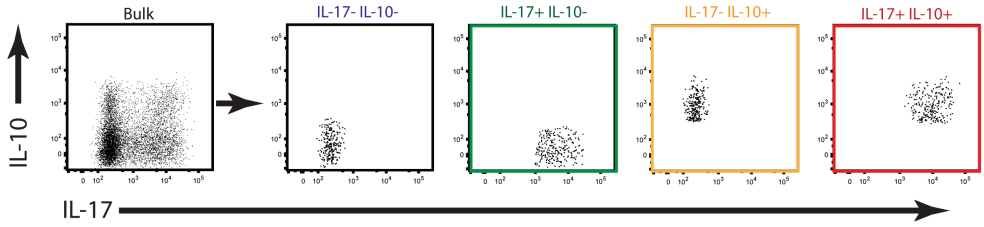
Oas3	*Scd1*	*Socs2*	*Tmc3*
Padi3	*Scd3*	*Sox21*	*Tmem154*
Pcdh7	*Selenbp1*	*Specc1*	*Tmem205*
Pde11a	*Selenbp2*	*Speer1-ps1*	*Tmem82*
Pear1	*Sell*	*Srrm4*	*Tnfrsf8*
Pik3cg	*Selp*	*St8sia1*	*Tns1*
Pik3ip1	*Sema3c*	*Stac2*	*Trat1*
Plcd1	*Sema4a*	*Stc2*	*Treml2*
Prdm1	*Serpinb1a*	*Styk1*	*Tshz2*
Procr	*Serpinb5*	*Susd3*	*Ubash3a*
Ptges	*Sh2d3c*	*Syk*	*Vill*
Ptprk	*Sh3d19*	*Sytl1*	*Wdfy4*
Rassf2	*Sh3pxd2b*	*Tcf7l2*	*Wwtr1*
Rimkla	*Siah3*	*Tet1*	*Xdh*
Rnase4	*Slc6a19*	*Tfap2a*	*Zfp365*
Rtkn2	*Slco4c1*	*Tg*	*Zfp608*
S1pr1	*Smox*	*Tgm2*	*Zpbp2*
Sash3	*Soat2*	*Tjp1*	*Zscan10*

A. **Th17 + anti-IL-2 Cells**



Cell Type	Population	Repeat 1	Repeat 2	Repeat 3
Th17 + anti-IL-2	Bulk	100.0	100.0	100.0
Th17 + anti-IL-2	IL-17- IL-10-	25.4	38.7	44.0
Th17 + anti-IL-2	IL-17+ IL-10-	57.4	49.5	44.6

B. **Th17 + IL-2 Cells**



Cell Type	Population	Repeat 1	Repeat 2	Repeat 3
Th17 + IL-2	Bulk	100.0	100.0	100.0
Th17 + IL-2	IL-17- IL-10-	49.5	62.1	54.2
Th17 + IL-2	IL-17+ IL-10-	21.0	9.9	13.5
Th17 + IL-2	IL-17+ IL-10+	8.5	6.5	8.5
Th17 + IL-2	IL-17- IL-10+	8.7	14.3	16.2

Figure 6B.1 Subpopulations of Th17 + IL-2 and Th17 + anti-IL-2 subsets

C57BL/6 naive CD4+CD62L+CD44loCD25- cells were driven with plate-bound anti-CD3 and soluble anti-CD28, and polarised to Th17 cells with IL-6, TGFβ, anti-IL-12, anti-IFNγ and anti-IL-4 in the presence or absence of IL-2 or anti-IL-2. Plots of sorted populations by flow cytometric analysis of intracellular cytokine staining. Tables showing the percentage of the total number of cells that each cytokine producing subpopulation represented for each of the three repeat experiments. The bulk is 100% of all CD4+ live cells as described in the Materials and Methods. **A.** Subpopulations within the Th17 + anti-IL-2 subset. **B.** Subpopulations within the Th7 + IL-2 subset.

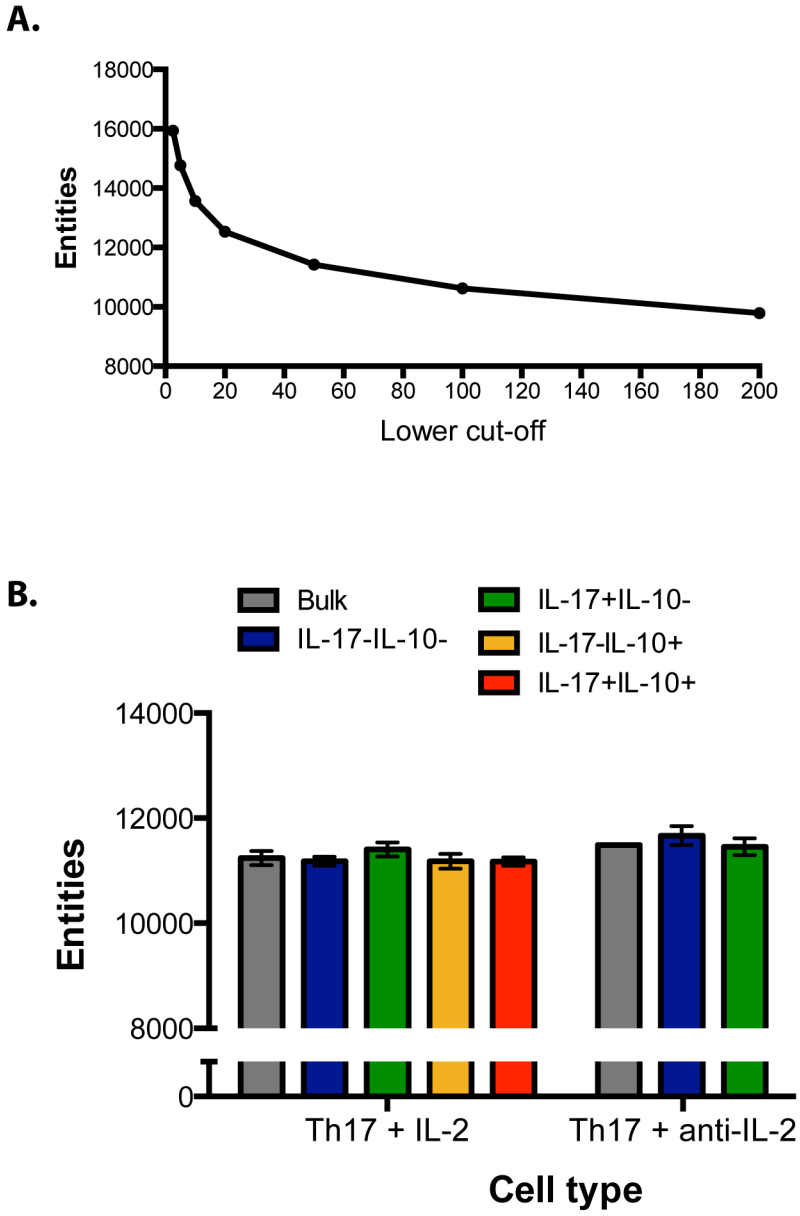


Figure 6B.2 Determining the optimal lower cut-off of reads for expression threshold of Th17 + IL-2 and Th17 + anti-IL-2 RNA-Seq samples

Cell populations are described in Figure 6B.1, mRNA extracted and prepared for RNA-Seq as described in Materials and Methods. Using Strand NGS software reads were aligned to the transcriptome & Genome (mm10, RefSeq annotation, 95% identity, max 5% gaps, 1 read only if duplicate) and normalisation with DeSeq and no Baseline. Upper cut-off 323885.938 (where at least 1 out of 24 samples have values within cut-off). **A.** The number of entities passing expression thresholds with indicated lower cut-offs. All the Th17 samples were pooled for this test. **B.** Using an expression threshold with a lower cut-off of 20 reads, the number of entities in each repeat of each subpopulation within the Th17 + IL-2 and Th17 + anti-IL-2 subsets was determined.

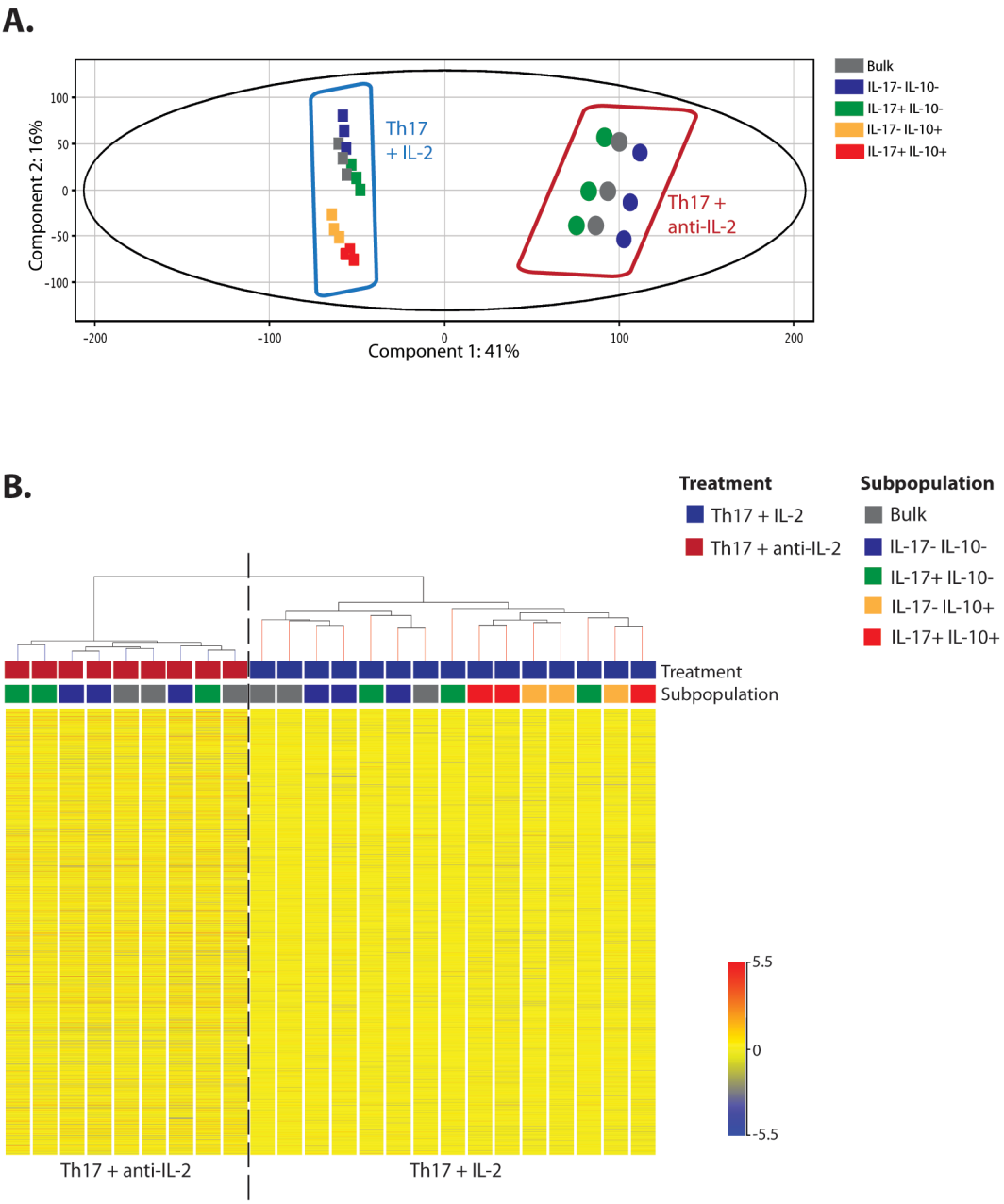


Figure 6B.3 PCA and cluster analysis demonstrates that Th17 + IL-2 and Th17 + anti-IL-2 cells have different gene expression profiles

Cell populations are described in Figure 6B.1, mRNA extracted and prepared for RNA-Seq as described in the Materials and Methods. **A.** PCA plots of intracellular cytokine producing subpopulations within the Th17 + IL-2 and Th17 + anti-IL-2 samples analysed by RNA-Seq. **B.** Unsupervised hierarchical clustering on conditions, of all individual repeats within the Th17 + IL-2 and Th17 + anti-IL-2 subsets, was carried out on the normalised intensity values with a Pearson's centred similarity measure and an Average linkage rule. Colour ranges indicate normalised intensity. The dendrogram represents the relatedness of the samples.

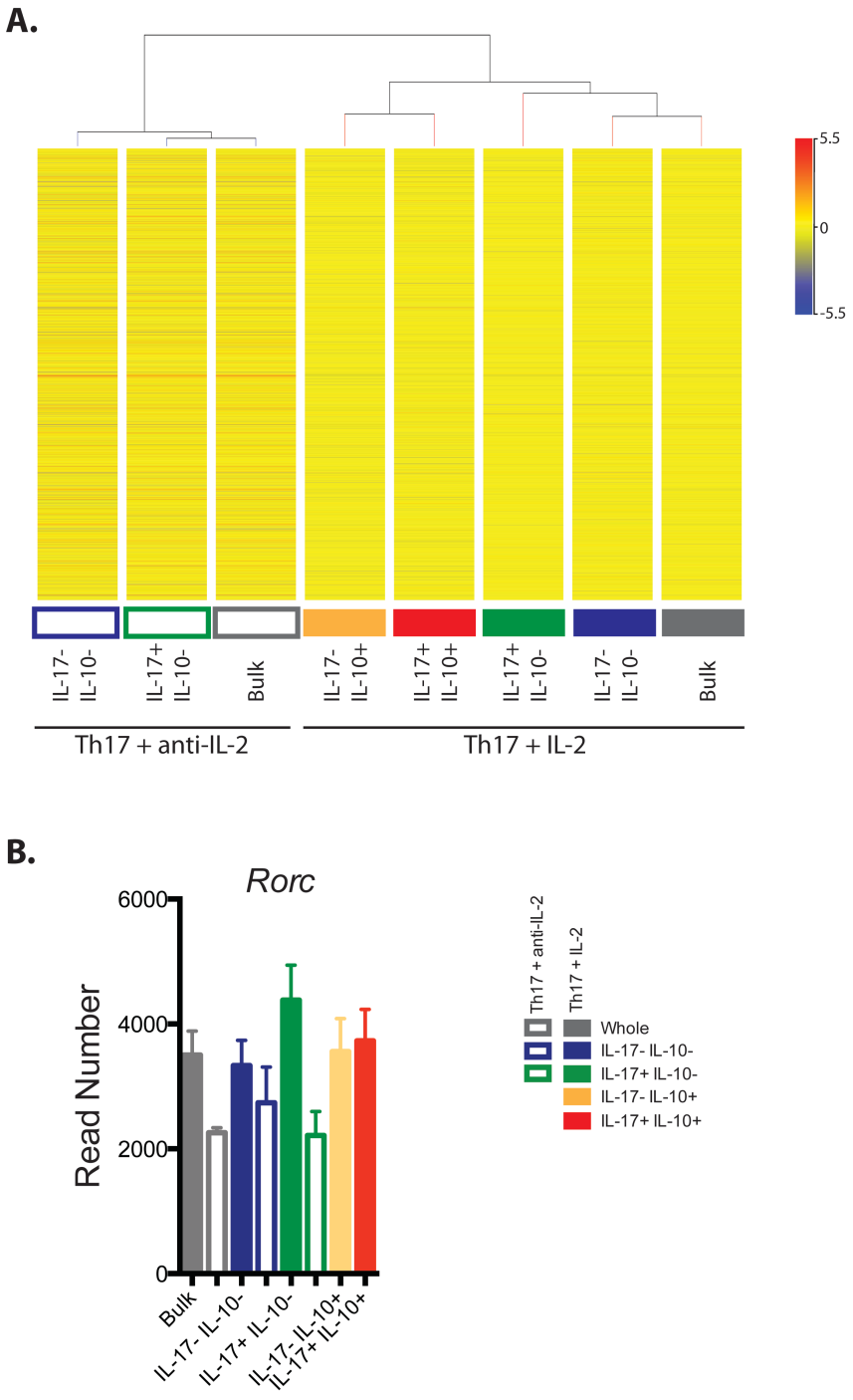


Figure 6B.4 Clustering demonstrates that Th17 + IL-2 and Th17 + anti-IL-2 cells are distinct and have transcriptional differences

Cell populations are described in Figure 6B.1, mRNA extracted and prepared for RNA-Seq as described in the Materials and Methods. Data from the three biological repeats were pooled. **A.** Unsupervised hierarchical clustering on conditions, of each subpopulations within the Th17 + IL-2 and Th17 + anti-IL-2 subsets, was carried out on the normalised intensity values with a Pearson's centred similarity measure and an Average linkage rule. Colour ranges indicate normalised intensity. The dendrogram represents the relatedness of the samples. **B.** The read number of *Rorc* in each subpopulation.

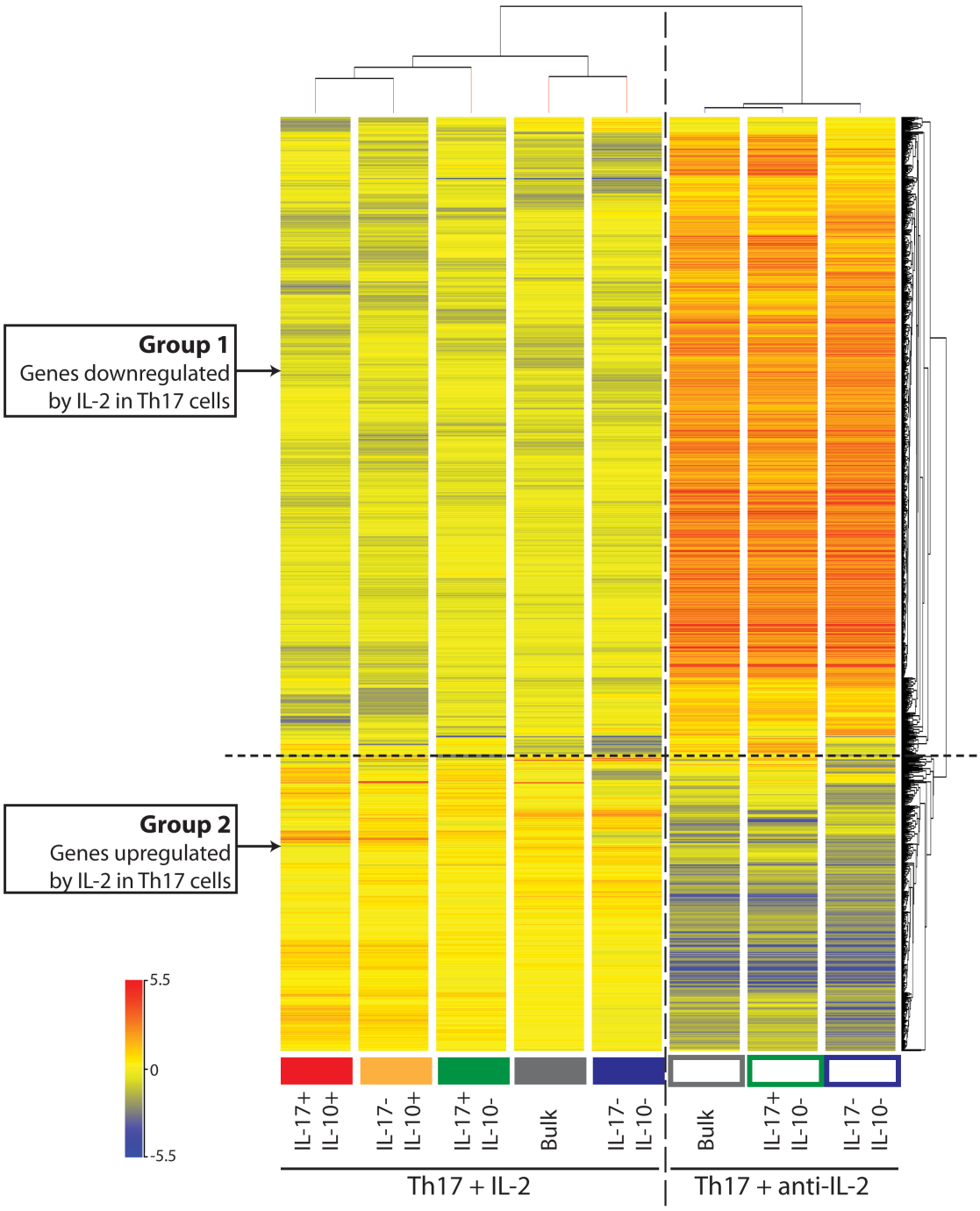
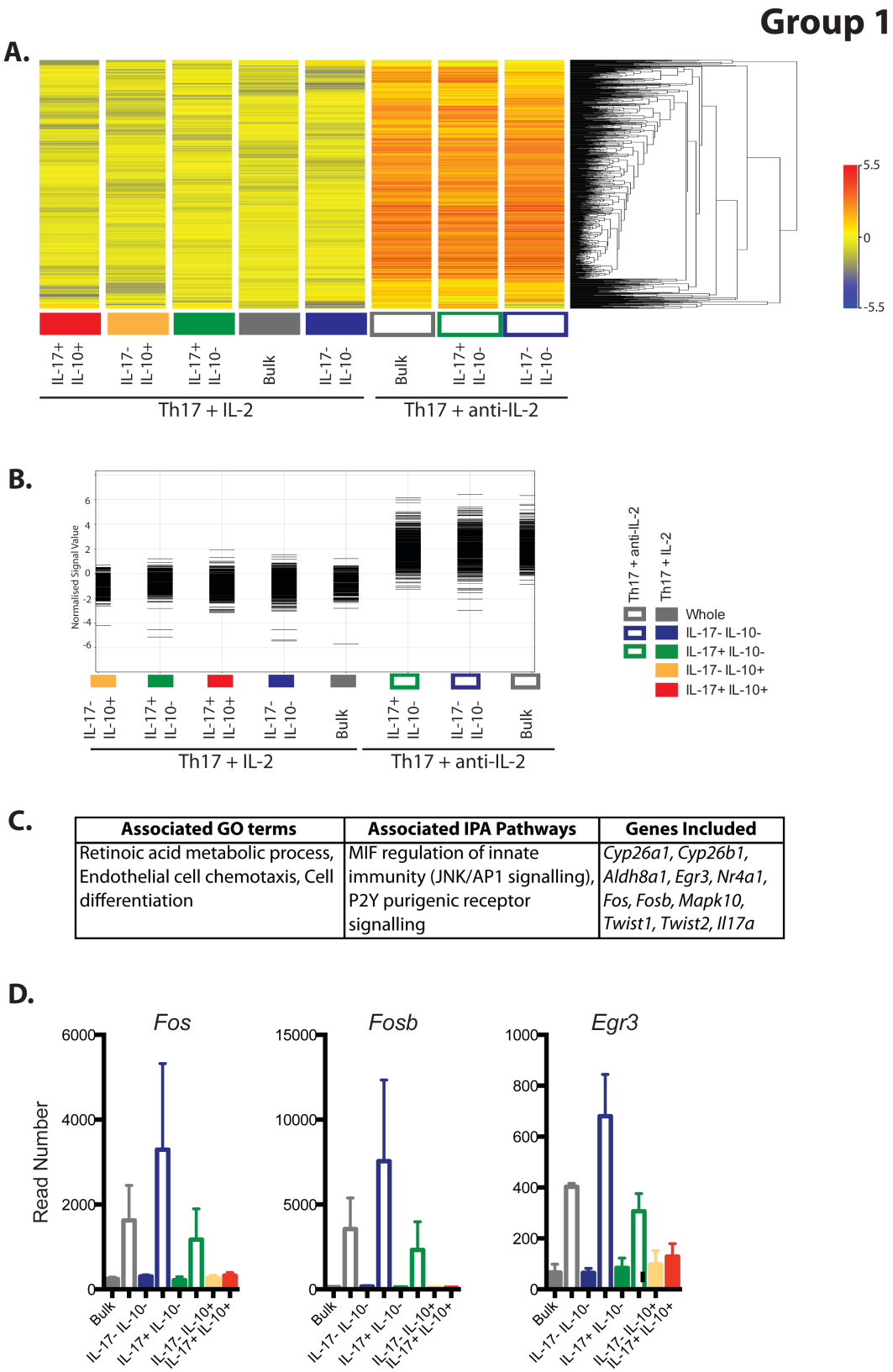


Figure 6B.5 There are dramatic transcriptional differences between the Th17 + IL-2 and Th17 + anti-IL-2 subsets

Cell populations are described in Figure 6B.1, mRNA extracted and prepared for RNA-Seq as described in the Materials and Methods. Data from the three biological repeats were pooled. Differentially regulated genes were obtained by taking those that were at least 3-fold up- or downregulated in at least 1 of the 8 samples vs. the baseline (median of all the samples). This left 1098 genes that were subjected to hierarchical clustering on entities and conditions with a Pearson's centred similarity measure and an Average linkage rule. Colour ranges indicate normalised intensity. The dendrogram represents the relatedness of the samples. Genes were separated into 2 groups based on the hierarchical dendrogram and experimental hypothesis.



Associated GO terms	Associated IPA Pathways	Genes Included
Retinoic acid metabolic process, Endothelial cell chemotaxis, Cell differentiation	MIF regulation of innate immunity (JNK/AP1 signalling), P2Y purigenic receptor signalling	*Cyp26a1, Cyp26b1, Aldh8a1, Egr3, Nr4a1, Fos, Fosb, Mapk10, Twist1, Twist2, Il17a*

Figure 6B.6.1 Group 1: 749 genes downregulated by IL-2 in Th17 cells

A. Genes in Group 1 from hierarchical clustering in Figure 6B.5. **B.** Expression profile of group. **C.** GO terms ($p<0.01$) and IPA pathways ($p<0.01$) with significant overlap, and examples of genes within the group. **D.** The read number of selected genes in each subpopulation.

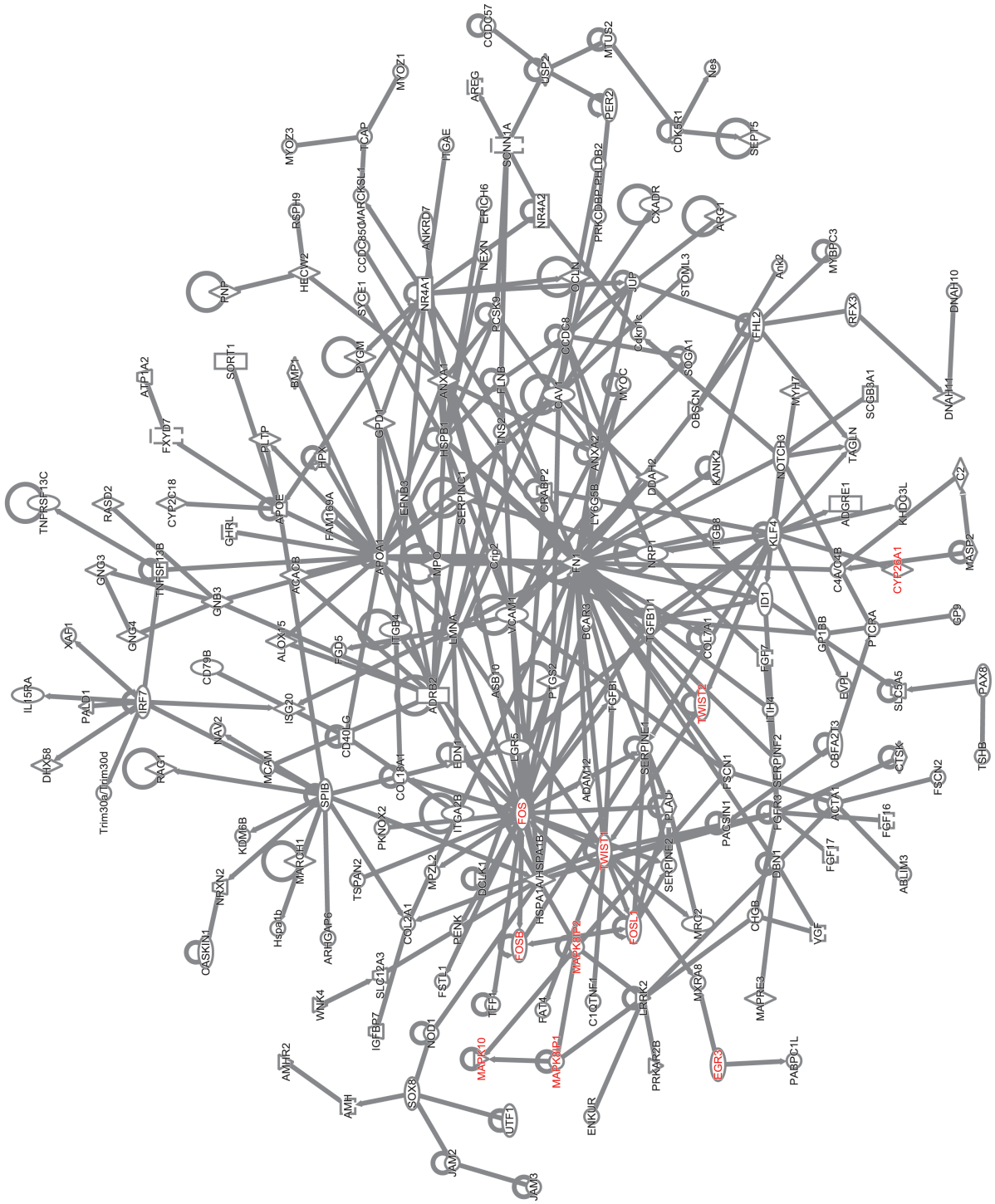


Figure 6B.6.2 Group 1: Network analysis of 749 genes downregulated by IL-2 in Th17 cells

IPA network analysis of direct interactions between genes. Genes in red are those discussed.

Table 6B.6.1 Group 1: List of 749 genes downregulated by IL-2 in Th17 cells

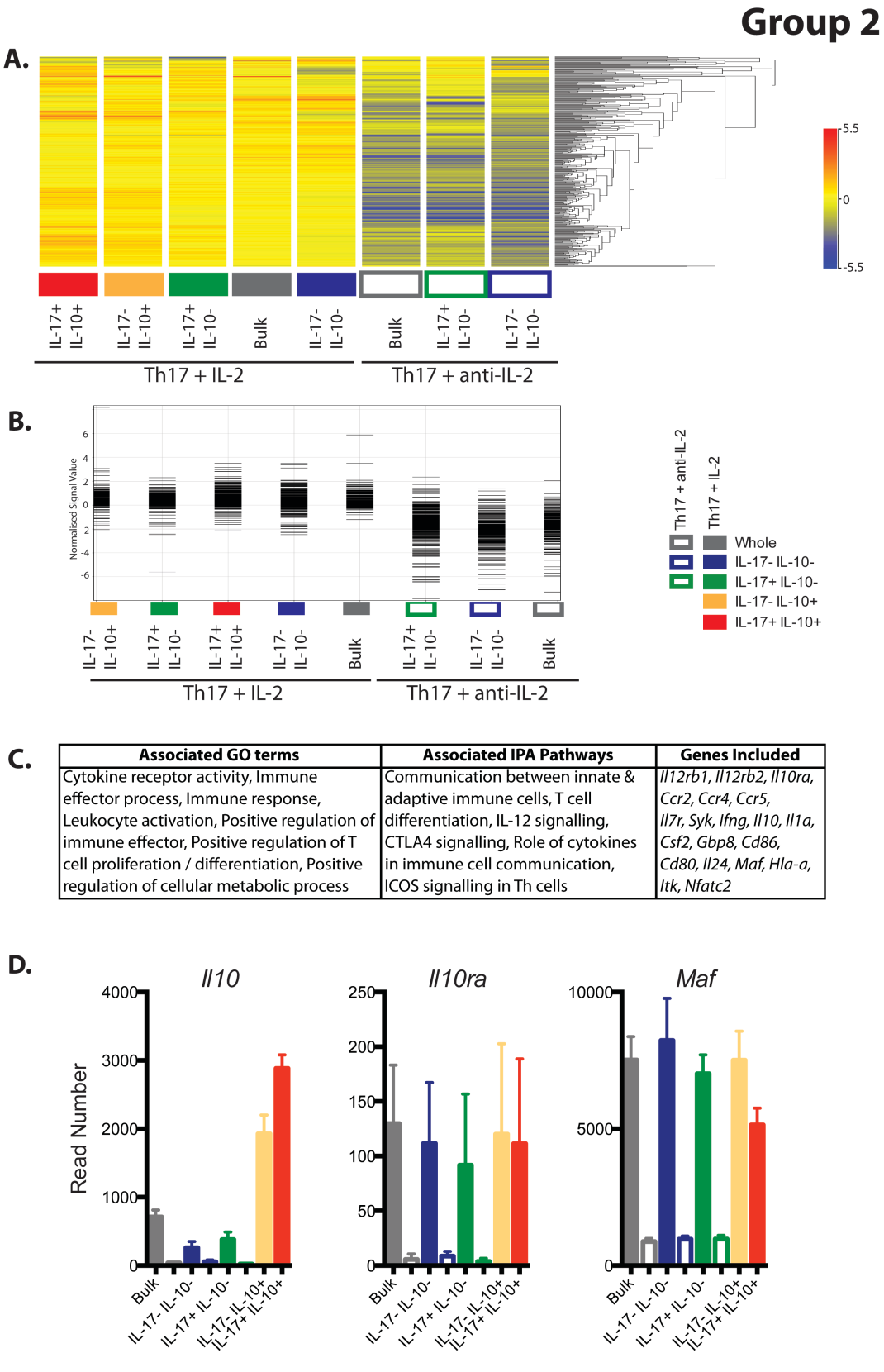
Genes highlighted in red are those also found in Group 1 of Table 6A.4.1.

0610009L18Rik	*9830001H06Rik*	*Arhgap40*	*Card9*
1190005I06Rik	*A3galt2*	*Arhgap6*	*Casc1*
1700001J03Rik	*A4galt*	*Arl4d*	*Caskin1*
1700001P01Rik	*AA986860*	*Arl9*	*Cav1*
1700003E16Rik	*Abcg4*	*Armc9*	*Cbfa2t3*
1700007K09Rik	*Ablim3*	*Arntl2*	*Ccdc11*
1700018B08Rik	*Acacb*	*Asb10*	*Ccdc151*
1700029J07Rik	*Acot12*	*Asgr1*	*Ccdc169*
1700094J05Rik	*Acpt*	*Atoh8*	*Ccdc28b*
1700102P08Rik	*Acta1*	*Atp1a2*	*Ccdc42b*
1700112J05Rik	*Actl7a*	*Atp6v0d2*	*Ccdc57*
1810010D01Rik	*Actl7b*	*Atp6v0e2*	*Ccdc73*
1810019D21Rik	*Adam12*	*Atp6v1b1*	*Ccdc74a*
1810043G02Rik	*Adam30*	*Avil*	*Ccdc8*
1810046K07Rik	*Adhfe1*	*B230216G23Rik*	*Ccdc85c*
2310002J15Rik	*Adig*	*B3galt2*	*Ccdc96*
2310014L17Rik	*Adra2a*	*B3gnt4*	*Ccin*
2410004P03Rik	*Adra2b*	*B3gnt6*	*Cckar*
2510049J12Rik	*Adrb2*	*Baat*	*Ccl25*
2810442I21Rik	*Agbl1*	*BC089491*	*Ccr8*
4921536K21Rik	*AI414108*	*BC100451*	*Cd164l2*
4930415F15Rik	*AI836003*	*Bcar3*	*Cd24a*
4930428O21Rik	*Aifl1*	*Bdkrb1*	*Cd40lg*
4930502E09Rik	*Ak7*	*Best2*	*Cd59b*
4930506M07Rik	*Akr1c18*	*Bicd1*	*Cd5l*
4930525D18Rik	*Aldh8a1*	*Bmp1*	*Cd70*
4930538K18Rik	*Alox15*	*Bnipl*	*Cd72*
4930548H24Rik	*Amh*	*Bok*	*Cd79b*
4930550C14Rik	*Amhr2*	*Bpifc*	*Cd96*
4930562C15Rik	*Ampd1*	*Bsn*	*Cd97*
4930590J08Rik	*Ank2*	*Bzrap1*	*Cdk5r1*
4931429I11Rik	*Ankrd13d*	*C1qtnf1*	*Cdkn1c*
4933404O12Rik	*Ankrd35*	*C1qtnf5*	*Cdo1*
4933405L10Rik	*Ankrd42*	*C1qtnf9*	*Cecr6*
4933407L21Rik	*Ankrd7*	*C2*	*Cel*
4933408B17Rik	*Anxa1*	*C4a*	*Celf5*
4933413G19Rik	*Anxa2*	*C530008M17Rik*	*Celsr2*
4933413J09Rik	*Aoc3*	*Cabp5*	*Celsr3*
4933422A05Rik	*Aph1c*	*Cacna1g*	*Chadl*
4933432I09Rik	*Apoa1*	*Cacna1s*	*Chd5*
5730577I03Rik	*Apoe*	*Calcb*	*Chgb*
5830411N06Rik	*Aqp7*	*Camkv*	*Chrm1*
6030408B16Rik	*Areg*	*Capn3*	*Chrnb2*
6330403K07Rik	*Arg1*	*Car15*	*Cib2*
6430550D23Rik	*Arg2*	*Car7*	*Cilp2*

Clcn1
Cldn15
Clec2i
Clec4a1
Cnksr1
Col12a1
Col18a1
Col25a1
Col2a1
Col7a1
Col9a3
Cplx1
Cplx3
Cpne9
Cpxm1
Crabp2
Crip2
Crtam
Ctrl
Ctsk
Ctxn1
Cuzd1
Cxadr
Cybrd1
Cyp26a1
Cyp26b1
Cyp2c55
Cyp2t4
Cyp39a1
Cyr61
D330023K18Rik
D4Ertd617e
D630029K05Rik
D6Ertd527e
Daam2
Dact3
Dbn1
Dclk1
Ddah2
Ddn
Depdc7
Dgki
Dhrs3
Dhrs9
Dhx58
Diras1
Dixdc1
Dlc1
Dlgap1
Dll3
Dmpk
Dnahc10
Dnahc11
Dnajc6
Dok3
Dusp18
Dyrk4
E530011L22Rik
Ebf1
Ecm2
Edn1
Efcab4a
Efcab5
Efcab9
Efhd1
Efna2
Efnb3
Egr3
Egr4
Elavl3
Elmod3
Emr1
Enkur
Enpp2
Epb4.1l4a
Epb4.1l5
Epb4.9
Epha8
Ephx3
Ermap
Erp27
Esam
Espnl
Esrp2
Evpl
Fam101a
Fam131a
Fam154b
Fam160a1
Fam167a
Fam169a
Fam171a2
Fam194a
Fam43a
Fam78b
Fam81a
Fam83e
Fat4
Fbxo2
Fcer1g
Fermt2
Fes
Fgd5
Fgf16
Fgf17
Fgf7
Fgfr3
Fgfrl1
Fhl2
Flnb
Fmnl2
Fn1
Fndc4
Fndc8
Fos
Fosb
Fosl1
Foxo6
Fscn1
Fscn2
Fscn3
Fst
Fstl1
Fxyd7
Gal3st1
Galr2
Gbx1
Gdf1
Gdpd2
Gfra1
Ghrl
Gja10
Gjc2
Gldc
Glt28d2
Gm10069
Gm1123
Gm1141
Gm11517
Gm128
Gm13718
Gm14085
Gm15708
Gm16197
Gm16853
Gm17455
Gm17745
Gm514
Gm5544
Gm766
Gm996
Gnb3
Gng3
Gng4
Gnmt
Gnrh1
Gp1bb
Gp9
Gpd1
Gpr124
Gpr152
Gpr153
Gpr3
Gpr35
Gpr44
Gprc5b
Greb1l
Grin2c
Grin3a
Gsta4
Gstt3
Guca1b
Gucy1a3
Gzmm
Hal
Hapln4
Hddc3
Hecw2
Henmt1
Hhatl
Hhipl2
Hist1h3a
Hpx
Hr
Hsd17b13
Hspa1a
Hspa1b
Hspb1
Hspb9
Htra4
Hyal3
Id1

Idi2
Ifi203
Igdcc4
Igfals
Igfbp7
Igj
Igsf9
Il15ra
Il17a
Il17c
Il17rb
Il17rc
Irf7
Isg20
Itga2b
Itgae
Itgb4
Itgb8
Itih4
Jam2
Jam3
Jsrp1
Jup
Kank2
Kcnc1
Kcnh4
Kcnh5
Kcnj14
Kcnk10
Kcnk4
Kdm6b
Khdc3
Kif17
Klf4
Klhl30
Kncn
L3mbtl1
Larp6
Lbx2
Lct
Lctl
Lgr5
Lin7b
Lipg
Lman1l
Lmna
Loxl3
Lrrc34
Lrrd1
Lrrk2
Lrrn4cl
Ltb4r2
Lum
Ly6g5b
Macc1
Mall
Map3k6
Map3k9
Mapk10
Mapk8ip1
Mapk8ip2
Mapre3
Mar1
Marcksl1
Masp2
Mboat4
Mc1r
Mc5r
Mcam
Mettl7a1
Mex3a
Mfrp
Mfsd2b
Mir24-2
Mn1
Mndal
Morn3
Mospd4
Mpo
Mpp4
Mpzl2
Mrc2
Mslnl
Mst1r
Mtus2
Muc1
Murc
Mxra8
Mybpc3
Myh3
Myh7
Myo15
Myo1a
Myo1e
Myoc
Myoz1
Myoz3
Naalad2
Naaladl1
Nacc2
Nav1
Nav2
Ncald
Ncan
Nccrp1
Nes
Neurog2
Nexn
Nfe2l3
Ngb
Nhlh1
Nkg7
Nod1
Notch3
Nox1
Noxo1
Npas4
Npc1l1
Npm2
Npr1
Nqo1
Nr4a1
Nr4a2
Nrgn
Nrn1
Nrn1l
Nrp1
Nrxn2
Ntn3
Nudt17
Nup210l
Nup62cl
Nxf7
Oas1b
Obscn
Ocln
Olfm2
Olfr536
Olfr56
Olfr60
Omg
Opn1sw
Opn3
Osr2
Otoa
Otop1
Otx1
Ovgp1
P2rx2
Pabpc1l
Pacsin1
Pak6
Pald1
Palld
Pax8
Pcsk1
Pcsk9
Pdcd1lg2
Pdlim4
Pdzd3
Pdzrn3
Penk
Per2
Phldb2
Pianp
Pip5kl1
Pknox2
Pla2g4b
Pla2g4c
Pla2g4f
Plau
Plcd4
Plce1
Pld4
Plekhd1
Plekhg4
Pllp
Pltp
Plxnc1
Pnp2
Pnpla1
Poln
Pon3
Pou3f1
Pou6f1
Ppfia4
Ppil6
Ppp1r32
Ppp1r3g
Prkar2b
Prkcdbp
Prnd

Proca1
Proz
Prr7
Prrt1
Prss46
Psmb11
Psors1c2
Ptch2
Ptcra
Ptgs2
Ptk6
Ptpn14
Pvrl1
Pvrl4
Pygm
Pyroxd2
Qrfp
Rab13
Rab36
Rag1
Rasd1
Rasd2
Rasl10a
Rasl11a
Rbp4
Rbpms2
Rdh1
Rdh9
Rec8
Rem2
Rep15
Rfx3
Rhbdf1
Rho
Rnf150
Rnf223
Rnf39
Rps6kl1
Rsph9
Scd4
Scgb3a1
Scin
Scn4a
Scnn1a
Sec16b
Sema4c
Sep5
Serpinc1
Serpine1
Serpine2
Serpinf2
Sez6l2
Sfrp2
Sh2d4b
Sh2d5
Sh3bgr
Shd
Shisa3
Shisa4
Slc12a3
Slc16a8
Slc17a6
Slc22a13
Slc23a3
Slc27a5
Slc2a5
Slc34a3
Slc39a10
Slc4a5
Slc5a2
Slc5a5
Slc6a17
Slc8a2
Slit1
Smad6
Smagp
Smpd3
Socs3
Sort1
Sox8
Sp7
Spem1
Spib
Spns2
Ssc5d
Sspo
Sstr2
Stk30
Stk36
Stoml3
Susd2
Syce1
Syngr4
Synpo2
Syt7
Tagln
Tagln3
Tas1r2
Tbr1
Tcap
Tcfl5
Tcp11
Tdrd6
Tecta
Tekt2
Tenc1
Tex15
Tff1
Tgfb1i1
Tgfbi
Thbs3
Thsd1
Tigd3
Timd2
Timm8a2
Tktl1
Tlr13
Tm6sf2
Tmed6
Tmem176a
Tmem176b
Tmem178
Tmem236
Tmem239
Tmem30c
Tmem44
Tmem52
Tmem88
Tmem91
Tmod1
Tmod4
Tmprss3
Tmprss9
Tnfrsf13c
Tnfsf13b
Tnn
Tnnt3
Tppp
Tppp3
Trh
Trim10
Trim15
Trim30d
Trim72
Tshb
Tspan2
Tssk1
Tssk2
Tssk3
Ttbk1
Ttc25
Ttc36
Tub
Tuba8
Twist1
Twist2
Ucn2
Unc13b
Upb1
Usp2
Utf1
Vash2
Vcam1
Vgf
Vil1
Vipr2
Vmn1r32
Vwa5b2
Vwa7
Vwce
Wasf1
Wnk4
Xaf1
Xcl1
Xrra1
Zan
Zbtb32
Zdhhc23
Zfand4
Zfp457
Zfp651
Zfp811
Zfp819
Zglp1
Zmynd10



Associated GO terms	Associated IPA Pathways	Genes Included
Cytokine receptor activity, Immune effector process, Immune response, Leukocyte activation, Positive regulation of immune effector, Positive regulation of T cell proliferation / differentiation, Positive regulation of cellular metabolic process	Communication between innate & adaptive immune cells, T cell differentiation, IL-12 signalling, CTLA4 signalling, Role of cytokines in immune cell communication, ICOS signalling in Th cells	*Il12rb1, Il12rb2, Il10ra, Ccr2, Ccr4, Ccr5, Il7r, Syk, Ifng, Il10, Il1a, Csf2, Gbp8, Cd86, Cd80, Il24, Maf, Hla-a, Itk, Nfatc2*

Figure 6B.7.1 Group 2: 349 genes upregulated by IL-2 in Th17 cells

A. Genes in Group 2 from hierarchical clustering in Figure 6B.5. **B.** Expression profile of group. **C.** GO terms ($p<0.05$) and IPA pathways ($p<0.01$) with significant overlap, and examples of genes within the group. **D.** The read number of selected genes in each subpopulation.

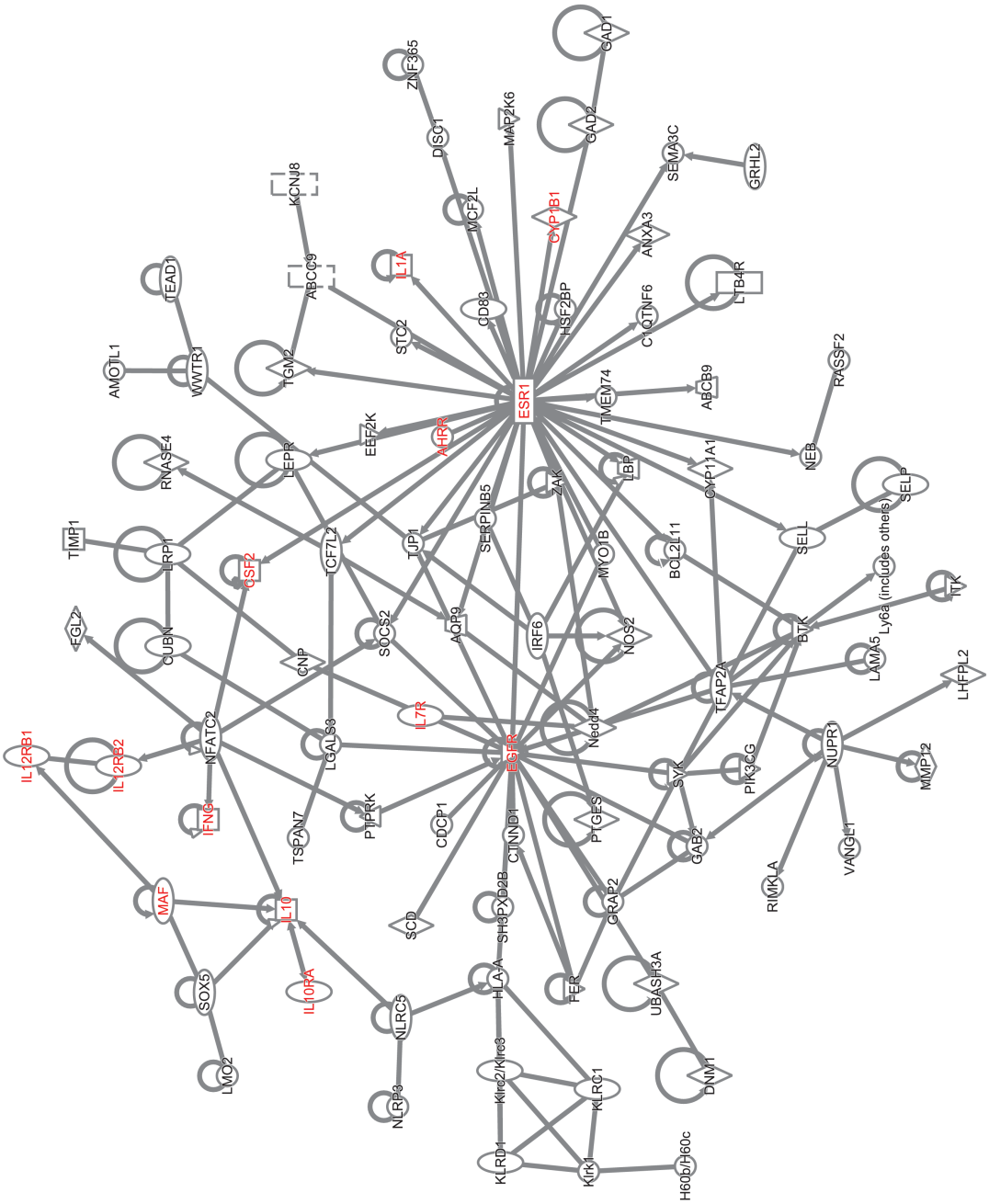


Figure 6B.7.2 Group 2: Network analysis of 349 genes upregulated by IL-2 in Th17 cells

IPA network analysis of direct interactions between genes. Genes in red are those discussed.

Table 6B.7.1 Group 2: List of 349 genes upregulated by IL-2 in Th17 cells

Genes highlighted in red are those also found in Group 1 of Table 6A.4.1. Genes highlighted in blue are those also found in Group 2 of Table 6A.4.2.

1110054M08Rik	*Asb2*	*Dleu7*	*Gm10390*
1500017E21Rik	*Auts2*	*Dnahc8*	*Gm10865*
1700066B19Rik	*B230120H23Rik*	*Dnm1*	*Gm11346*
1700097N02Rik	*B430306N03Rik*	*Dntt*	*Gm11413*
1700120C14Rik	*Batf3*	*E130218I03Rik*	*Gm12185*
2010005H15Rik	*BC055111*	*Ebi3*	*Gm12709*
2210408F21Rik	*Bcl2l11*	*Ecm1*	*Gm13032*
2900011O08Rik	*Bcl2l14*	*Eda2r*	*Gm13546*
4930452B06Rik	*Btbd8*	*Eef2k*	*Gm14005*
4930486L24Rik	*Btk*	*Efcab11*	*Gm16548*
4930539E08Rik	*C1qtnf6*	*Egfr*	*Gm17384*
4932438H23Rik	*C430002N11Rik*	*Enpp1*	*Gm17801*
5031414D18Rik	*Cacna1h*	*Enthd1*	*Gm20098*
5033411D12Rik	*Cass4*	*Entpd1*	*Gm4827*
5830416P10Rik	*Ccr2*	*Epn2*	*Gm5122*
6430571L13Rik	*Ccr4*	*Ermn*	*Gm5483*
6430706D22Rik	*Ccr5*	*Esr1*	*Gm6455*
9430060I03Rik	*Cd200r1*	*Exd1*	*Gm6460*
9930111J21Rik1	*Cd200r4*	*F2rl2*	*Gm7030*
A130049A11Rik	*Cd226*	*Faim3*	*Gm867*
A630023P12Rik	*Cd80*	*Fam110c*	*Gp49a*
A830010M20Rik	*Cd83*	*Fam151a*	*Gpc3*
A930004D18Rik	*Cd86*	*Fam183b*	*Gphb5*
AA467197	*Cd93*	*Fam71b*	*Gpr114*
Abcb9	*Cdcp1*	*Fanci*	*Gpr141*
Abcc9	*Cdh17*	*Fert2*	*Gpr146*
Abcg3	*Clmn*	*Ffar2*	*Gpr15*
Acot11	*Cnp*	*Fggy*	*Gpr55*
Acsl6	*Cox4i2*	*Fgl2*	*Gpr97*
Adamtsl5	*Cox6a2*	*Folr4*	*Grap2*
Adcy4	*Cpn2*	*Foxf2*	*Grhl2*
Ahrr	*Cpne6*	*Fstl4*	*Grm6*
AI661453	*Csf2*	*Fut10*	*Grtp1*
Aldh1l2	*Ctla2a*	*Fut7*	*H2-Q1*
Alpk2	*Ctla2b*	*Fzd10*	*H60b*
Amotl1	*Ctnnd1*	*Gab2*	*Hemk1*
Ampd3	*Cubn*	*Gad1*	*Hepacam2*
Ang	*Cwh43*	*Gad2*	*Hist1h1a*
Angptl6	*Cyp11a1*	*Gap43*	*Hmx2*
Anxa3	*Cyp1b1*	*Gbp8*	*Hmx3*
Aqp9	*Cysltr1*	*Gimap7*	*Hoxd8*
Arap3	*D730005E14Rik*	*Gipr*	*Hsf2bp*
Arhgap19	*Ddx25*	*Gja5*	*Ifitm3*
Arhgap25	*Disc1*	*Gli3*	*Ifng*

Il10
Il10ra
Il12rb1
Il12rb2
Il1a
Il24
Il3
Il7r
Ildr1
Inha
Irf6
Itga7
Itgax
Itih5
Itk
Kbtbd13
Kcnj8
Klrc1
Klrc2
Klrc3
Klrd1
Klre1
Klri2
Klrk1
Kremen2
Krt222
Lama5
Lbp
Lepr
Lgals3
Lhfpl2
Lhx6
Lilrb4
Lmo2
LOC100503496
Lrfn3
Lrp1
Lrrc32
Lrrc66
Ltb4r1
Ly6a
Ly6c1
Ly6i
Lzts1
Maf
Map2k6
Mcf2l
Mctp2
Micall2
Mlkl
Mmp12
Mmp24
Mnd1
Msi1
Mt1
Mt2
Muc4
Myo16
Myo1b
Myo1f
Myo3b
Myof
Naip2
Nanog
Nap1l5
Ndnf
Neb
Nedd4
Nfatc2
Nhs
Nlrc5
Nlrp3
Nodal
Nos2
Nr1i2
Nupr1
Oas3
Obsl1
Osbpl1a
Otogl
Padi3
Palm2
Pbx1
Pcdh7
Pde11a
Pdpn
Pik3cg
Pik3ip1
Pkd2
Plk5
Pnma3
Prf1
Procr
Ptges
Ptpn5
Ptprk
Ptprn
Rassf2
Rimkla
Rnase4
Rnf180
Rnf217
S1pr1
S1pr3
Sash3
Scd1
Selenbp1
Selenbp2
Sell
Selp
Sema3c
Sema4a
Serpinb1a
Serpinb5
Serpinf1
Sgce
Sgip1
Sgtb
Sh3d19
Sh3pxd2b
Siah3
Slc6a19
Smox
Smtn
Snai3
Sncb
Soat2
Socs2
Sorcs3
Sox5
Sp9
Specc1
Speer1-ps1
Srrm4
St8sia1
Stac2
Stc2
Stfa2
Stfa2l1
Styk1
Syk
Sytl1
Tcf7l2
Tead1
Tet1
Tfap2a
Tg
Tgm2
Tifab
Timp1
Tjp1
Tmem154
Tmem205
Tmem40
Tmem74
Tmem82
Tnfrsf13b
Tnfrsf8
Tns1
Treml2
Tspan7
Ubash3a
Ugt1a7c
Vangl1
Vill
Wdfy4
Wwtr1
Xdh
Xpnpep2
Zfp133-ps
Zfp365
Zfp57
Zscan10

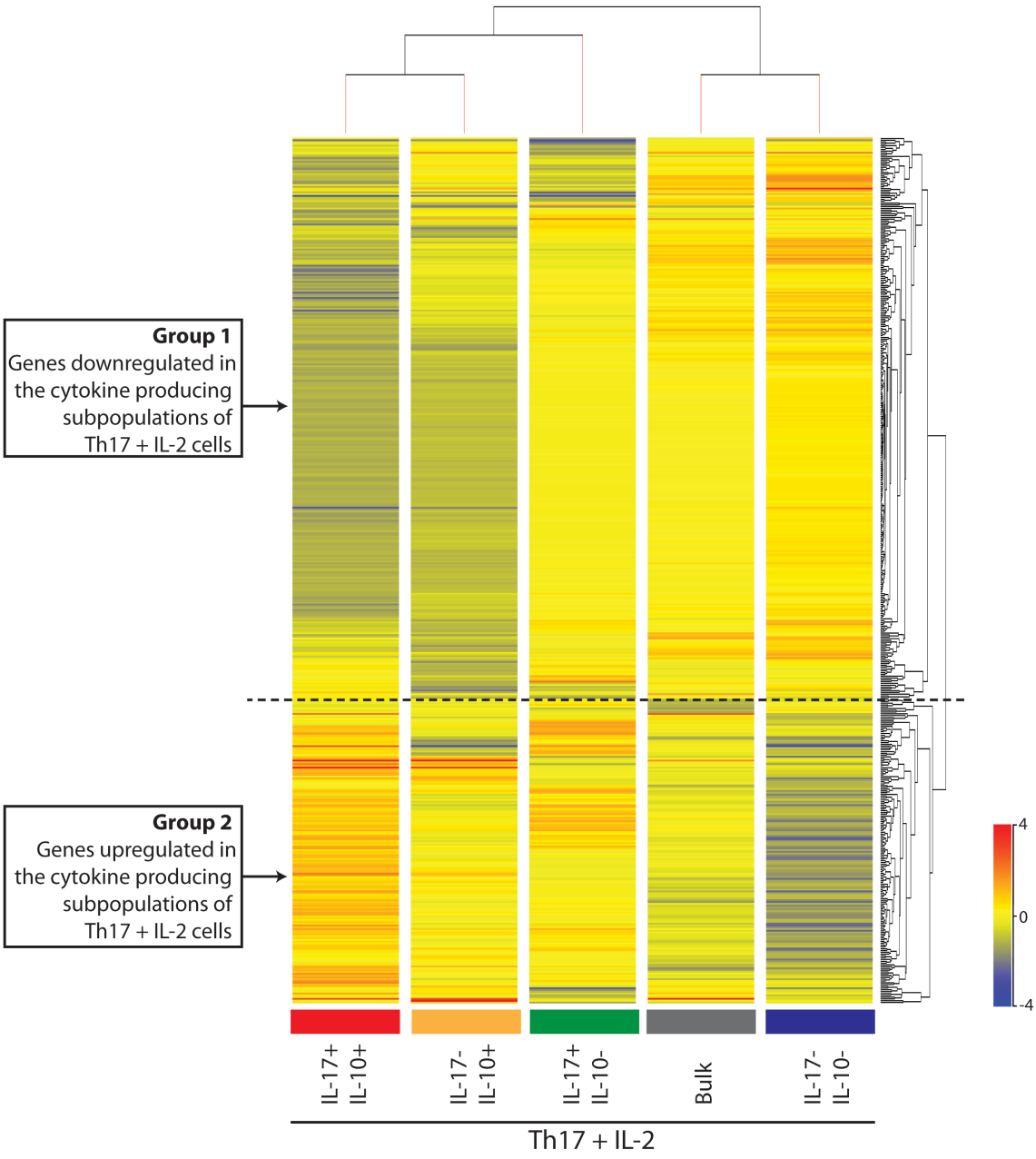


Figure 6B.8 There are dramatic transcriptional differences between the subpopulations within the Th17 + IL-2 subset

Cell populations are described in Figure 6B.1, mRNA extracted and prepared for RNA-Seq as described in the Materials and Methods. Data from the three biological repeats within the Th17 + IL-2 subset were pooled. Differentially regulated genes were obtained by taking those that were at least 2-fold up- or downregulated in at least 1 of the 5 samples vs. the baseline (median of all the samples). This left 483 genes that were subjected to hierarchical clustering on entities and conditions with a Pearson's centred similarity measure and an Average linkage rule. Colour ranges indicate normalised intensity. The dendrogram represents the relatedness of the samples. Genes were separated into 2 groups based on the hierarchical dendrogram and experimental hypothesis.

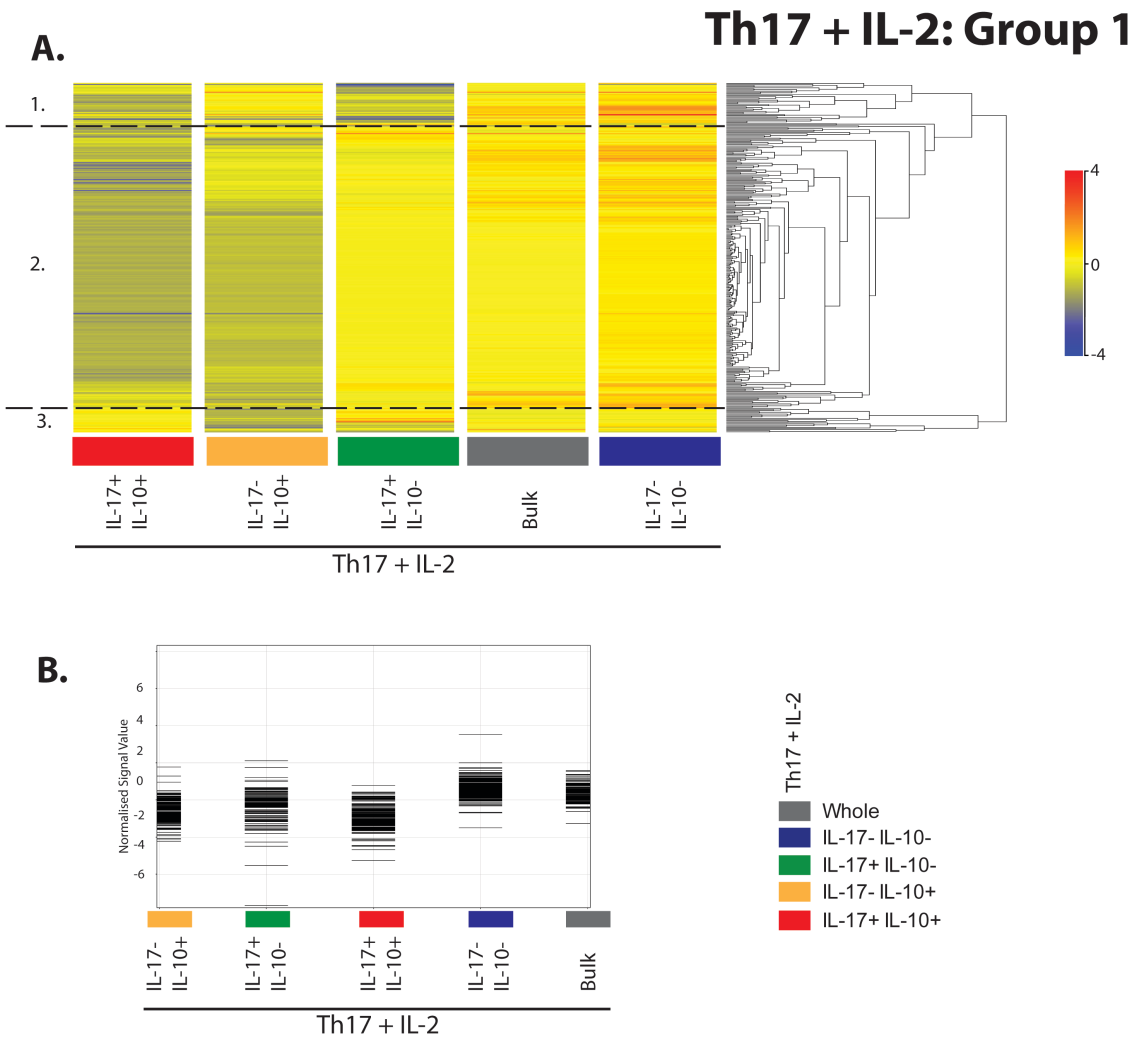


Figure 6B.9.1 Group 1: 313 genes downregulated in the cytokine producing subpopulations of the Th17 + IL-2 cells

A. Genes in Group 1 from hierarchical clustering in Figure 6B.8. **B.** Expression profile of group.

Table 6B.9.1 Group 1, List 1: 36 genes downregulated in the IL-17+ IL-10- and IL-17+ IL-10+ subpopulations of the Th17 + IL-2 cells

1500017E21Rik	*Calcb*	*Gm4532*	*Qrfp*
1700023L04Rik	*Cass4*	*Gpr83*	*Rab37*
1700058G18Rik	*Cxcl10*	*Grm6*	*Serpinb5*
1700120C14Rik	*F3*	*Hepacam2*	*Serpinc1*
9430060I03Rik	*Foxp3*	*Hrh2*	*Slco4c1*
A130049A11Rik	*Gbp4*	*Ifi203*	*Slfn1*
Akr1c18	*Gm10390*	*Itgb8*	*Tfap2a*
Arap3	*Gm10865*	*Mmp24*	*Tshz2*
Btk	*Gm14005*	*Plekhd1*	*Ttc23*

Th17 + IL-2: Group 1 - List 2

A.

Associated GO terms	Associated IPA Pathways	Genes Included
Cellular metabolic process, Cell cycle	Cell cycle	*Bard1, Cdk1, Ccnb1, Foxm1*

B.

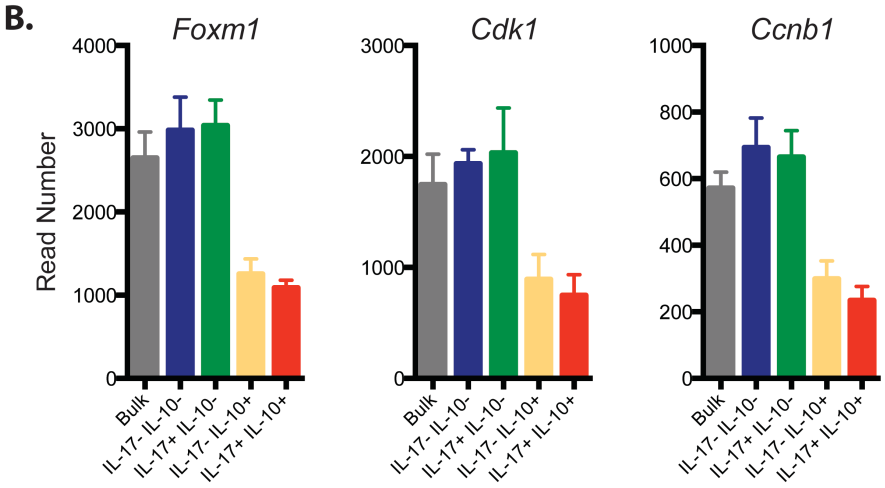


Figure 6B.9.2 Group 1, List 2: 255 genes downregulated in the IL-17- IL-10+ and IL-17+ IL-10+ subpopulations of the Th17 + IL-2 cells

A. GO terms ($p<0.01$) and IPA pathways ($p<0.01$) with significant overlap, and examples of genes within the group. **B.** The read number of selected genes in each subpopulation.

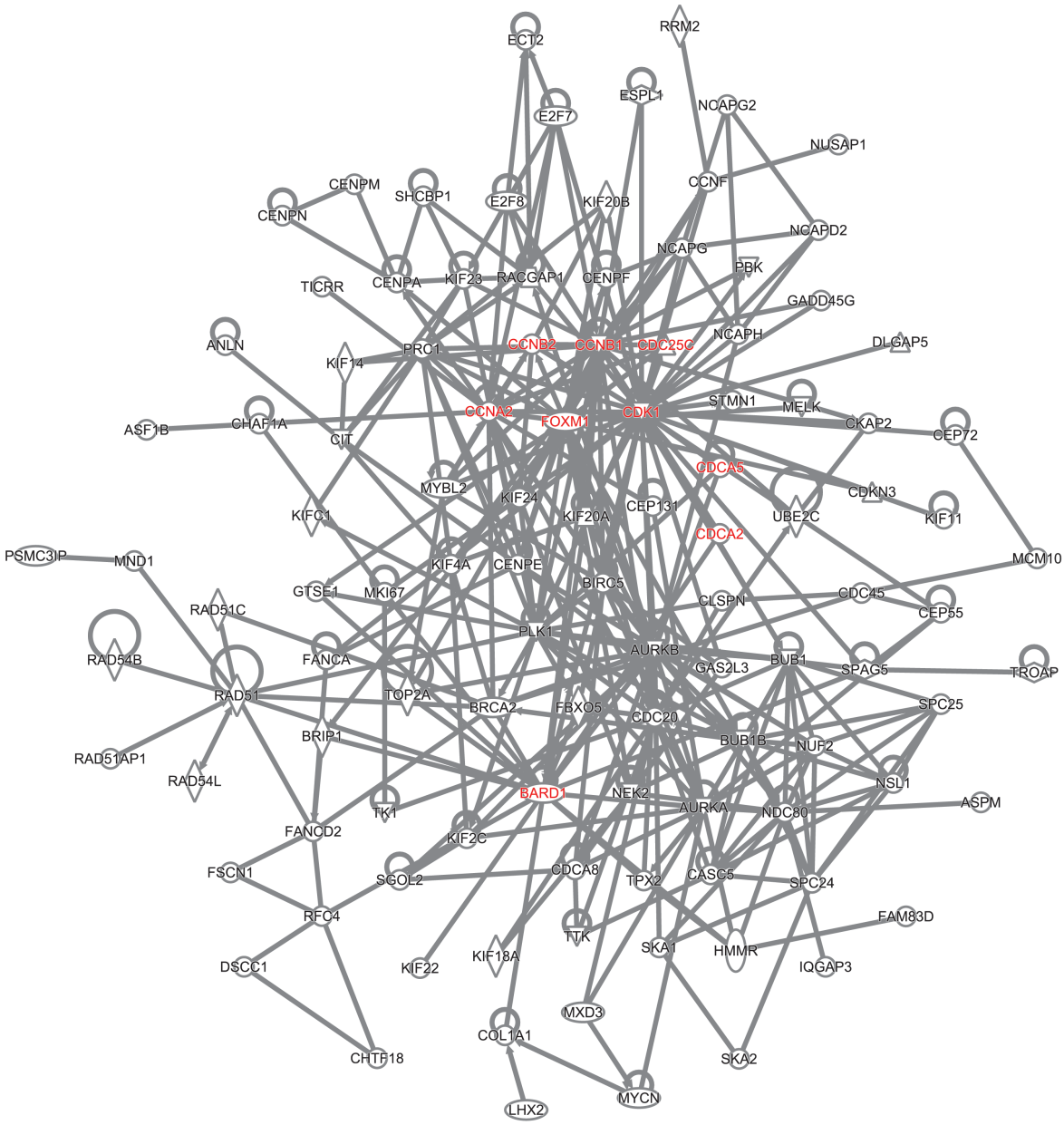


Figure 6B.9.3 Group 1, List 2: Network analysis 255 genes downregulated in the IL-17- IL-10+ and IL-17+ IL-10+ subpopulations of the Th17 + IL-2 cells

IPA network analysis of direct interactions between genes. Genes in red are those discussed.

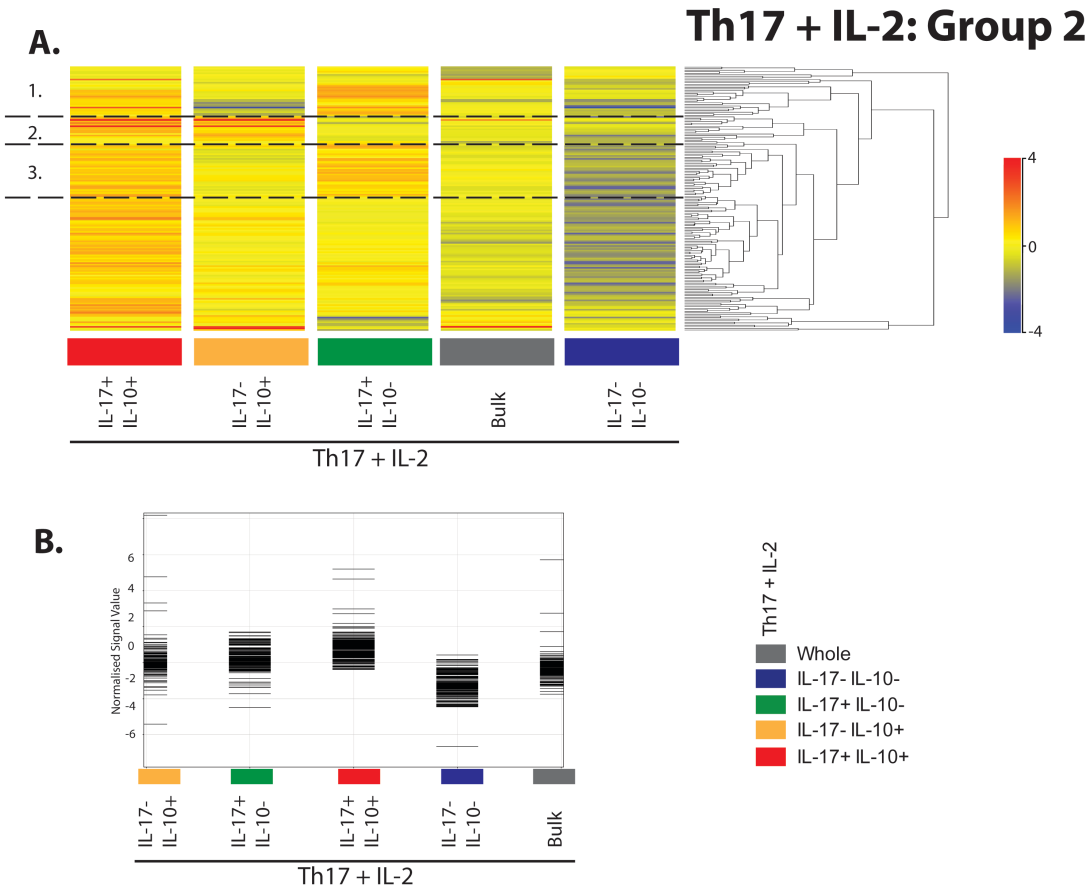
Table 6B.9.2 Group 1, List 2: 255 genes downregulated in the IL-17- IL-10+ and IL-17+ IL-10+ subpopulations of the Th17 + IL-2 cells

1190002F15Rik	*Cd109*	*Espl1*	*Kif18a*
2410004P03Rik	*Cdc20*	*Exo1*	*Kif18b*
2810417H13Rik	*Cdc25c*	*Fads2*	*Kif20a*
2810442I21Rik	*Cdc45*	*Fam64a*	*Kif20b*
4930558J18Rik	*Cdca2*	*Fam83d*	*Kif22*
4933404O12Rik	*Cdca3*	*Fanca*	*Kif23*
9530077C05Rik	*Cdca5*	*Fancd2*	*Kif24*
Acacb	*Cdca8*	*Fbxo2*	*Kif2c*
Adam12	*Cdk1*	*Fbxo5*	*Kif4*
Adcy9	*Cdk5r1*	*Fert2*	*Kifc1*
AI182371	*Cdkn3*	*Foxm1*	*Klf2*
Ankle1	*Cecr6*	*Fsbp*	*Kntc1*
Ankrd34b	*Cenpa*	*Fscn1*	*Lhx2*
Anln	*Cenpe*	*Fut10*	*Lhx6*
Apobec1	*Cenpf*	*Gadd45g*	*Lonrf3*
Arhgap19	*Cenpm*	*Gas2l3*	*Lrr1*
Arhgef39	*Cenpn*	*Gen1*	*Lrrc49*
Asf1b	*Cep55*	*Gins1*	*Mastl*
Aspm	*Cep72*	*Gm13718*	*Mcm10*
Aurka	*Chaf1a*	*Gm17745*	*Melk*
Aurkb	*Chtf18*	*Gm1966*	*Mettl7a1*
Azi1	*Cit*	*Gpr44*	*Mis18bp1*
Bambi-ps1	*Ckap2*	*Gpr55*	*Mki67*
Bard1	*Ckap2l*	*Gprc5b*	*Mnd1*
BC030867	*Clec2i*	*Gstt3*	*Mndal*
Birc5	*Clspn*	*Gtse1*	*Mxd3*
Brca2	*Col1a1*	*Gucy2e*	*Mybl2*
Brip1	*Cox6a2*	*H1fx*	*Mycn*
Bsn	*Cyp39a1*	*Herc6*	*Ncapd2*
Bspry	*Depdc1a*	*Hhipl2*	*Ncapg*
Bub1	*Depdc1b*	*Hist1h1b*	*Ncapg2*
Bub1b	*Dlgap5*	*Hist1h1d*	*Ncaph*
Cacnb1	*Dnajc6*	*Hist1h2bc*	*Ndc80*
Camkmt	*Drp2*	*Hist1h3a*	*Neil3*
Car7	*Dscc1*	*Hist1h3c*	*Nek2*
Casc5	*E130218I03Rik*	*Hmmr*	*Neurl1b*
Ccdc141	*E2f7*	*Hmx2*	*Nhs*
Ccdc18	*E2f8*	*Hspa1b*	*Npas4*
Ccdc34	*Ect2*	*Iqgap3*	*Nqo2*
Ccna2	*Efcab11*	*Irs1*	*Nrap*
Ccnb1	*Efcab5*	*Itih5*	*Nrarp*
Ccnb2	*Eme1*	*Kank2*	*Nsl1*
Ccnf	*Ephx1*	*Kif11*	*Nuf2*
Ccr8	*Esco2*	*Kif14*	*Nusap1*

Oas1c	*Psmc3ip*	*Shcbp1*	*Top2a*
Ogdhl	*Ptk7*	*Ska1*	*Tpx2*
Oip5	*Pvrl2*	*Ska2*	*Trat1*
Osbpl1a	*Racgap1*	*Skida1*	*Troap*
Osgin1	*Rad51*	*Smoc1*	*Tst*
Pbk	*Rad51ap1*	*Smpdl3b*	*Ttk*
Phf19	*Rad51c*	*Socs3*	*Ube2c*
Pif1	*Rad54b*	*Sp9*	*Vipr2*
Pla2g4c	*Rad54l*	*Spag5*	*Vsig10*
Plek2	*Rec8*	*Spc24*	*Wdr62*
Plk1	*Rfc4*	*Spc25*	*Wdr90*
Pmch	*Rimkla*	*St8sia4*	*Xkr5*
Poc1a	*Rnf223*	*Stil*	*Zan*
Pole	*Rpph1*	*Stmn1*	*Zfand4*
Pole2	*Rrm2*	*Syk*	*Zfp300*
Polq	*Rtkn2*	*Syngr4*	*Zfp879*
Ppargc1b	*Rtn4rl1*	*Tcf19*	*Zpbp*
Prc1	*S1pr1*	*Ticrr*	*Zranb3*
Prkar2b	*Sema4c*	*Tk1*	*Zwilch*
Prr11	*Sgol2*	*Tmem121*	

Table 6B.9.3 Group 1, List 3: 22 genes downregulated in the IL-17- IL-10+ subpopulation of the Th17 + IL-2 cells

1700102P08Rik	*Chit1*	*Gucy1a3*	*Myo5b*
Arntl2	*Cyp1b1*	*H2-Q1*	*Plcd3*
B230208H11Rik	*D630041G03Rik*	*Klf5*	*Slc8a1*
Batf3	*E230016M11Rik*	*Lrrn4cl*	*Tcp11*
Bicd1	*Gm17801*	*Mcam*	
Cd5l	*Gpr124*	*Mpp4*	



C.

Associated GO terms	Associated IPA Pathways	Genes Included
Cytokine receptor binding, Inflammatory response, Cellular biosynthetic processes, Positive regulation of metabolic process	Differential regulation of cytokine produciton, Communication between innate & adaptive immune cells, Fatty acid α-oxidation	*Il17f, Il10, Il1a, Cxcr3, Il3, Ccl3, Ccl4, Ccl5, Lmo2, Sox5, Ebf1, Il24*

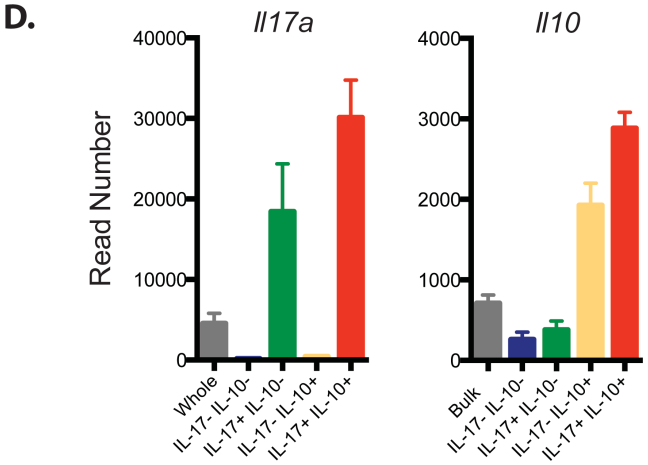


Figure 6B.10.1 Group 2: 170 genes upregulated in the cytokine producing subpopulations of the Th17 + IL-2 cells

A. Genes in Group 2 from hierarchical clustering in Figure 6B.8. **B.** Expression profile of group. **C.** GO terms ($p<0.01$) and IPA pathways ($p<0.01$) with significant overlap, and examples of genes within the group. **D.** The read number of selected genes in each subpopulation.

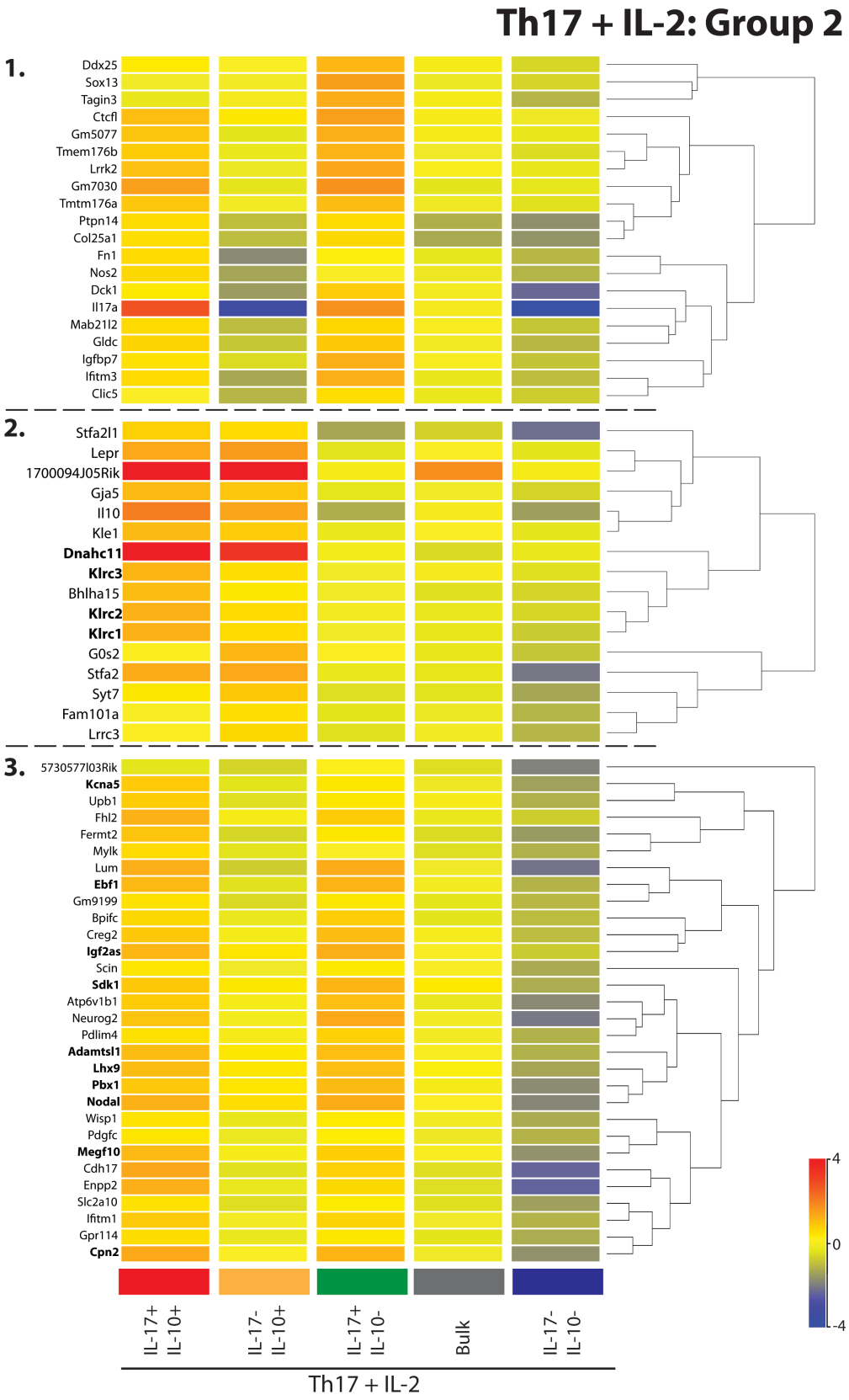


Figure 6B.10.2 Group 2: Further analysis of clusters of genes within the 170 genes upregulated in the cytokine producing subpopulations of the Th17 + IL-2 cells

3 lists of genes that form distinctive clusters within Group 2 from hierarchical clustering in Figure 6B.8. Genes were separated into 3 lists based on the hierarchical dendrogram and experimental hypothesis. Genes in bold are those discussed.

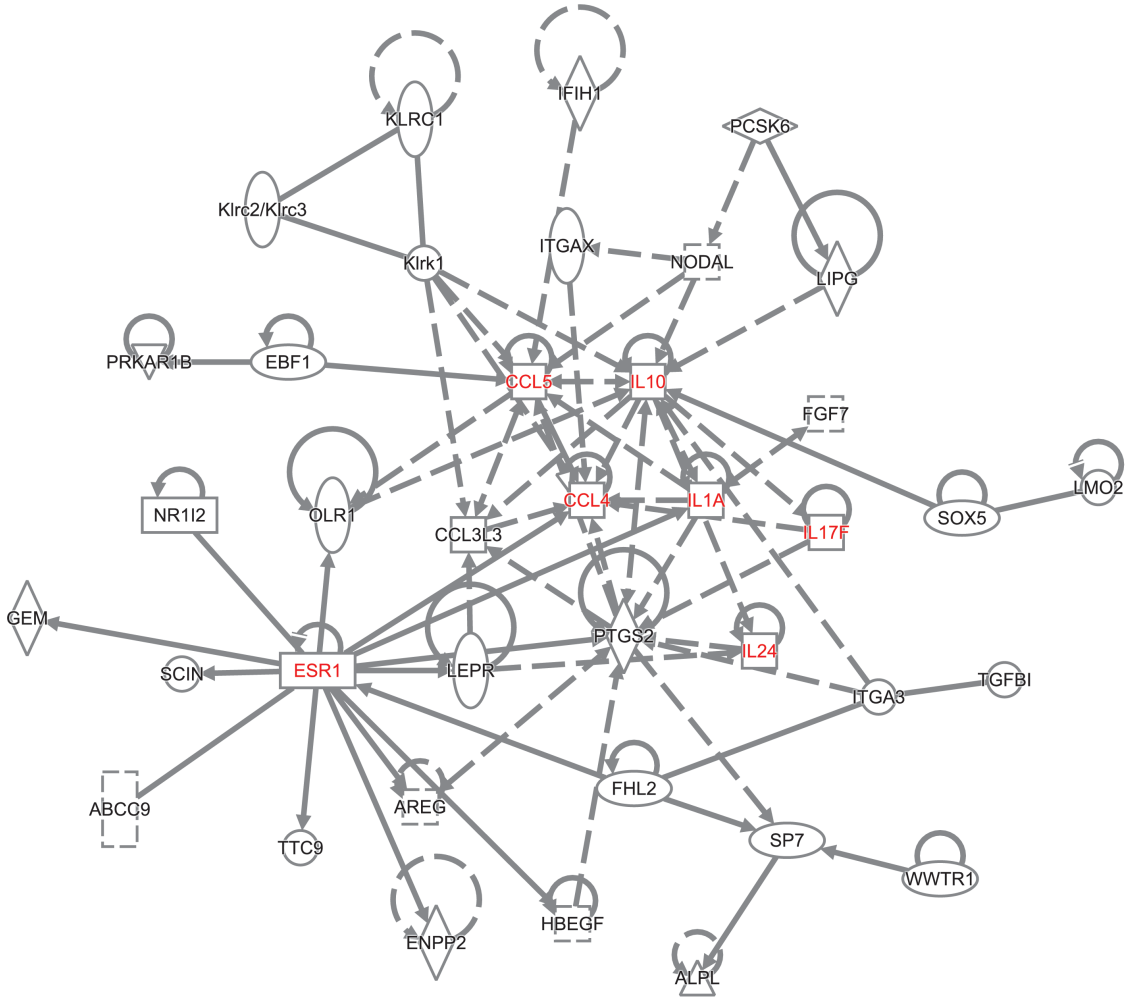


Figure 6B.10.3 Group 2: Network analysis of 170 genes upregulated in the cytokine producing subpopulations of the Th17 + IL-2 cells

IPA network analysis of direct and indirect interactions between genes. Solid lines indicate direct interactions, the dashed lines indicate indirect interactions. Genes in red are those discussed.

Table 6B.10.1 Group 2: List of 170 genes upregulated in the cytokine producing subpopulations of the Th17 + IL-2 cells

1700094J05Rik	*Ddx25*	*Il3*	*Ppp1r3g*
4930562F07Rik	*Dlc1*	*Impg2*	*Prkar1b*
4933416M07Rik	*Dnahc11*	*Irf7*	*Ptgs2*
5730577I03Rik	*Dysf*	*Ism1*	*Ptpn14*
6430706D22Rik	*Ebf1*	*Itga3*	*Ptprn*
A330009N23Rik	*Enpp2*	*Itgax*	*Rag1*
A730090N16Rik	*Ermn*	*Kcna5*	*Rgs8*
Abcc9	*Esr1*	*Kcnk10*	*Scin*
Abhd16b	*Fam101a*	*Kcnrg*	*Sdc2*
Adamtsl1	*Fermt2*	*Klrc1*	*Sdk1*
AI414108	*Ffar2*	*Klrc2*	*Sertm1*
Alox12b	*Fgf7*	*Klrc3*	*Shank1*
Alpl	*Fhl2*	*Klre1*	*Slc17a6*
Ano1	*Fn1*	*Klrk1*	*Slc2a10*
Areg	*G0s2*	*Lctl*	*Sncb*
Astn1	*Gad1*	*Lepr*	*Snora34*
Atp6v1b1	*Gdpd2*	*Lgr5*	*Sorcs3*
Bhlha15	*Gem*	*Lhx9*	*Sox13*
Bpifc	*Gfpt2*	*Lipg*	*Sox5*
C030016D13Rik	*Gja5*	*Lmo2*	*Sp7*
C130050O18Rik	*Gldc*	*Lrrc3*	*Stfa2*
C1qtnf1	*Gm11413*	*Lrrk2*	*Stfa2l1*
C430002N11Rik	*Gm13889*	*Lum*	*Stox2*
Cacna1h	*Gm17384*	*Mab21l2*	*Styk1*
Ccl3	*Gm5077*	*Megf10*	*Syt5*
Ccl4	*Gm7030*	*Mylk*	*Syt7*
Ccl5	*Gm9199*	*Myo16*	*Tagln3*
Cd8a	*Gpat2*	*Nanog*	*Tgfbi*
Cd93	*Gpr114*	*Neurog2*	*Thsd7a*
Cdcp1	*Gpr141*	*Nodal*	*Timd2*
Cdh17	*Gpr97*	*Nos2*	*Tmem176a*
Cdk18	*Hbegf*	*Nr1i2*	*Tmem176b*
Celsr3	*Hpn*	*Nrn1l*	*Tmem82*
Chadl	*Ifih1*	*Nts*	*Treh*
Clic5	*Ifitm1*	*Olr1*	*Ttc9*
Cnksr1	*Ifitm3*	*Otogl*	*Upb1*
Col25a1	*Igf2as*	*Palm2*	*Upk3bl*
Cpn2	*Igfbp7*	*Pbx1*	*Wisp1*
Creg2	*Il10*	*Pcsk6*	*Wwtr1*
Ctcfl	*Il17a*	*Pdgfc*	*Xcr1*
Cxcl3	*Il17f*	*Pdlim4*	*Zfp57*
Dclk1	*Il1a*	*Pglyrp1*	
Ddn	*Il24*	*Pnma3*	

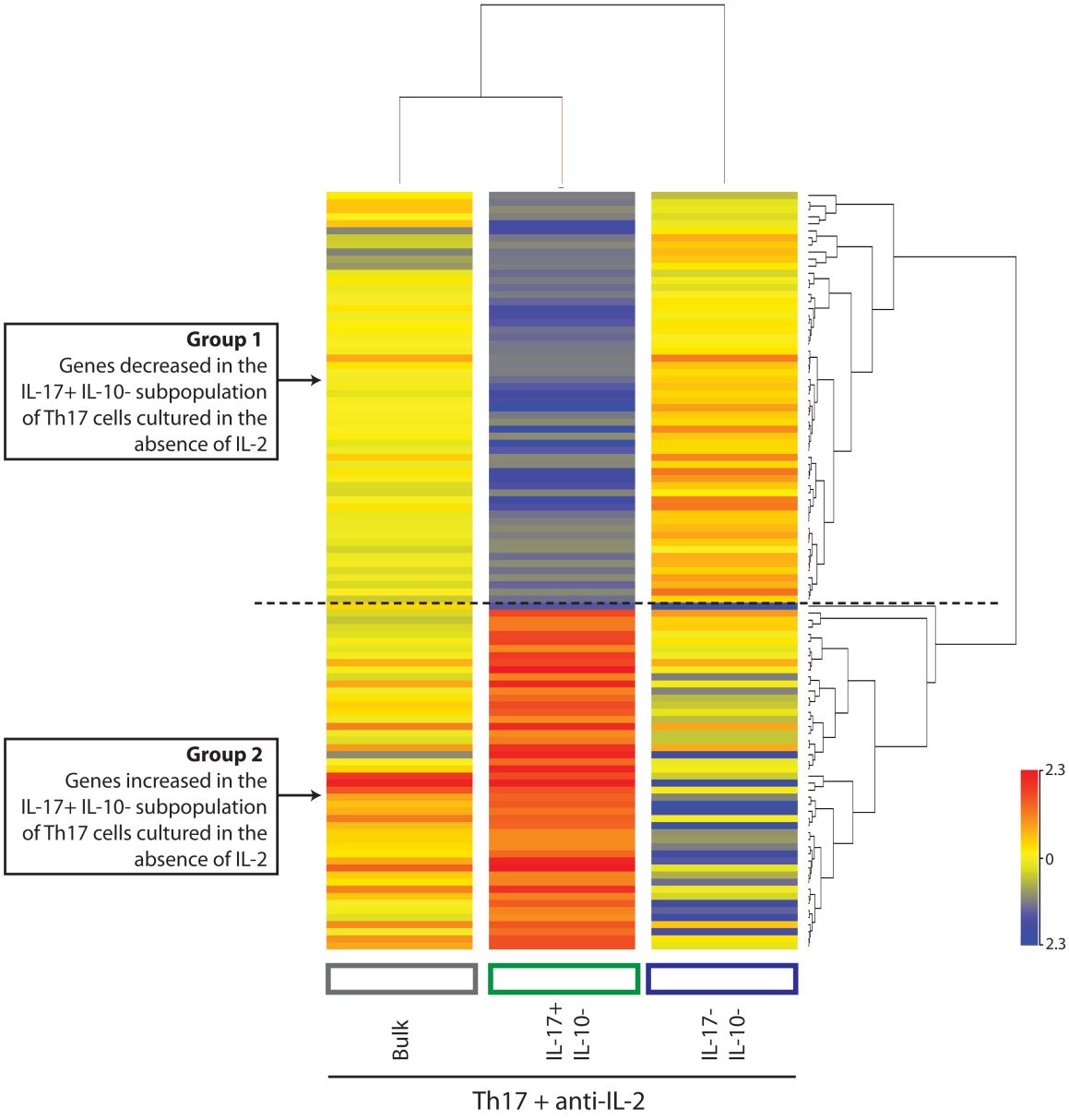
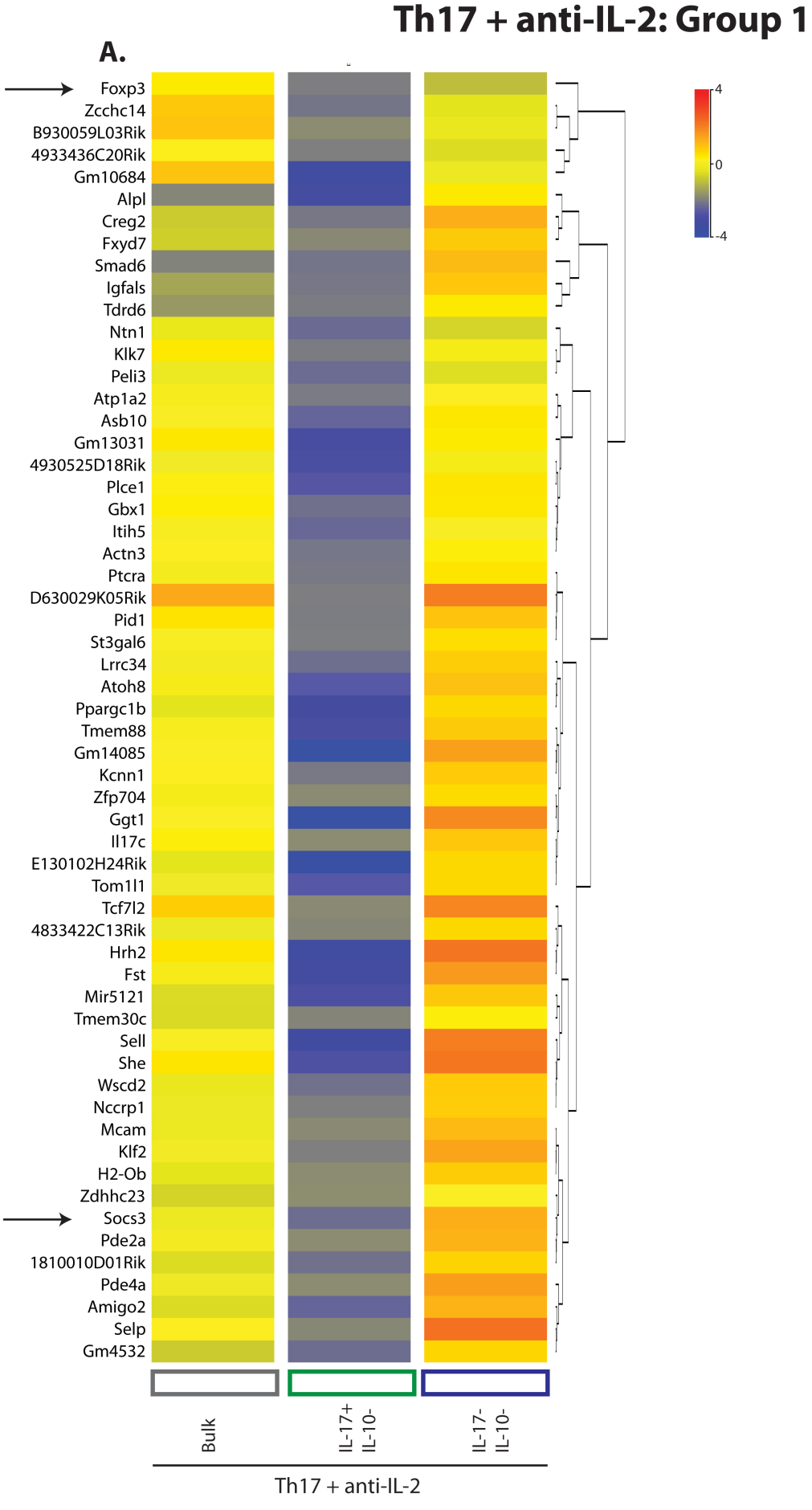
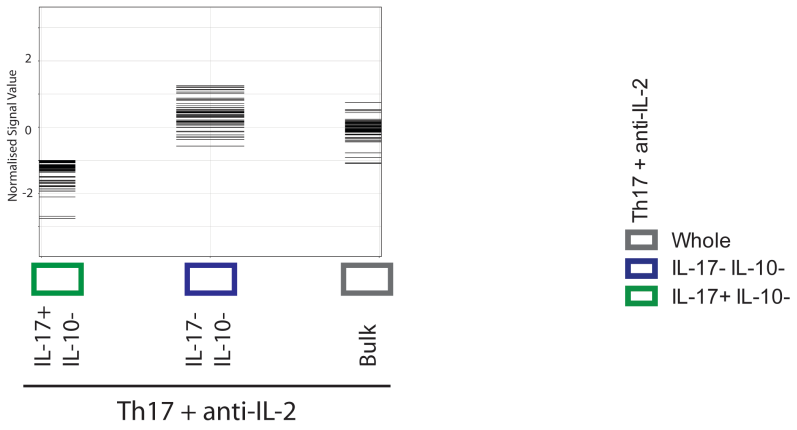


Figure 6B.11 There are dramatic transcriptional differences between the subpopulations within the Th17 + anti-IL-2 subset

Cell populations are described in Figure 6B.1, mRNA extracted and prepared for RNA-Seq as described in the Materials and Methods. Data from the three biological repeats within the Th17 + anti-IL-2 subset were pooled. Differentially regulated genes were obtained by taking those that were at least 2-fold up- or downregulated in at least 1 of the 3 samples vs. the baseline (median of all the samples). This left 106 genes that were subjected to hierarchical clustering on entities and conditions with a Pearson's centred similarity measure and an Average linkage rule. Colour ranges indicate normalised intensity. The dendrogram represents the relatedness of the samples. Genes were separated into 2 groups based on the hierarchical dendrogram and experimental hypothesis.


Th17 + anti-IL-2: Group 1
A.
Foxp3
Zcchc14
B930059L03Rik
4933436C20Rik
Gm10684
Alpl
Creg2
Fxyd7
Smad6
Igfals
Tdrd6
Ntn1
Klk7
Peli3
Atp1a2
Asb10
Gm13031
4930525D18Rik
Plce1
Gbx1
Itih5
Actn3
Ptcra
D630029K05Rik
Pid1
St3gal6
Lrrc34
Atoh8
Ppargc1b
Tmem88
Gm14085
Kcnn1
Zfp704
Ggt1
Il17c
E130102H24Rik
Tom1l1
Tcf7l2
4833422C13Rik
Hrh2
Fst
Mir5121
Tmem30c
Sell
She
Wscd2
Nccrp1
Mcam
Klf2
H2-Ob
Zdhhc23
Socs3
Pde2a
1810010D01Rik
Pde4a
Amigo2
Selp
Gm4532
4
0
-4
Bulk
IL-17+ IL-10-
IL-17- IL-10-
Th17 + anti-IL-2

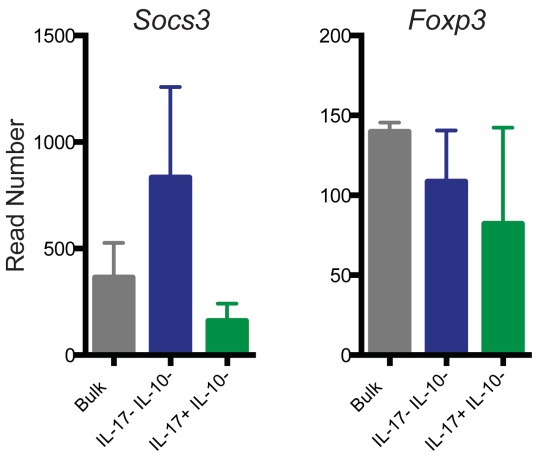
B.



C.

Associated GO terms	Associated IPA Pathways	Genes Included
Cellular response to mechanical stimulus	Protein Kinase A signalling, T helper cell differentiation	*Socs3, H2-Ob, Il17c, FoxP3, Smad6*

D.



E.

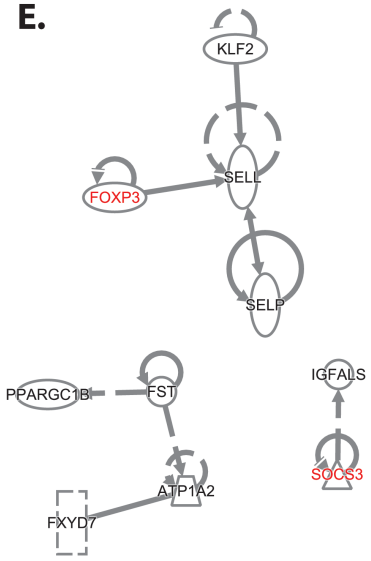
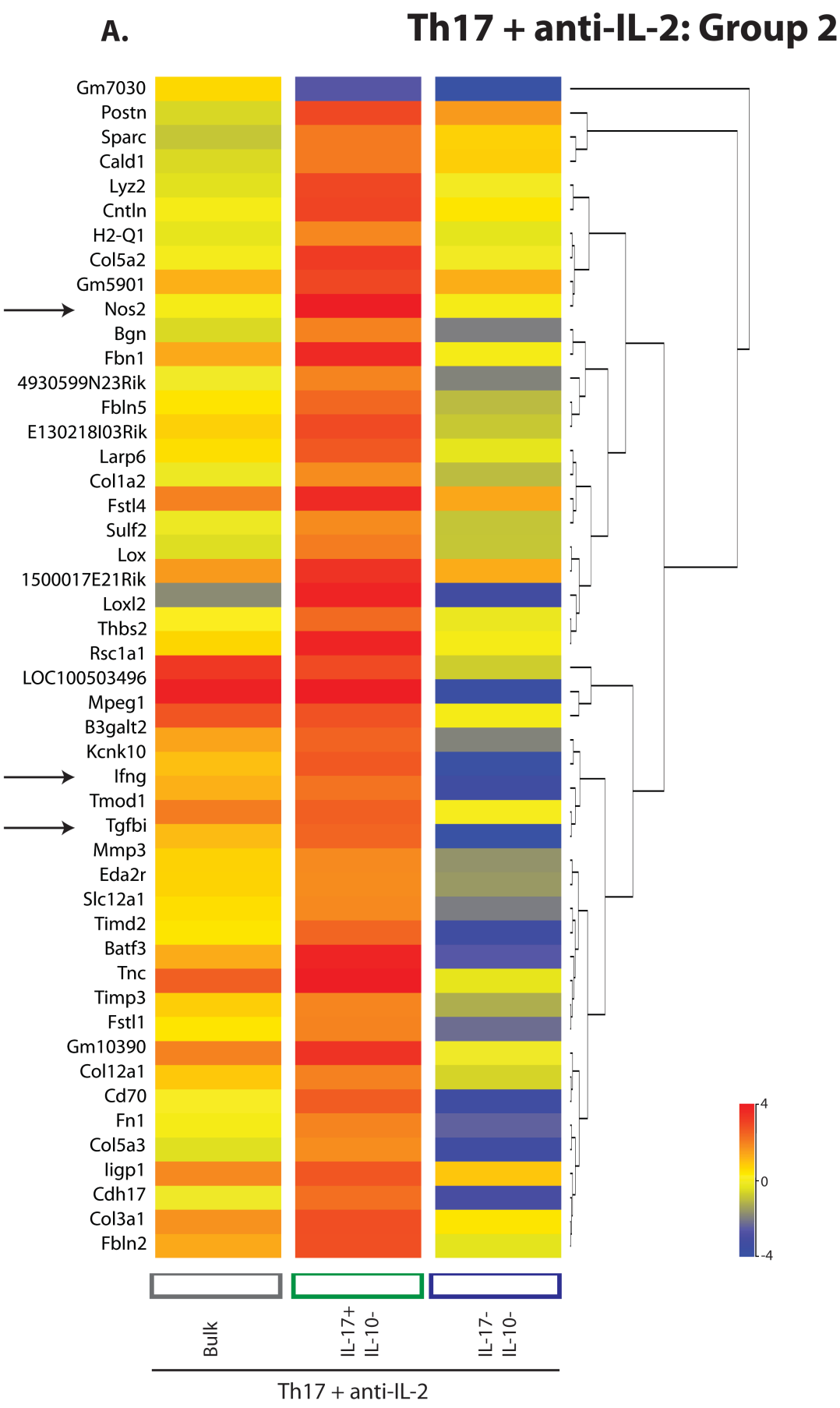
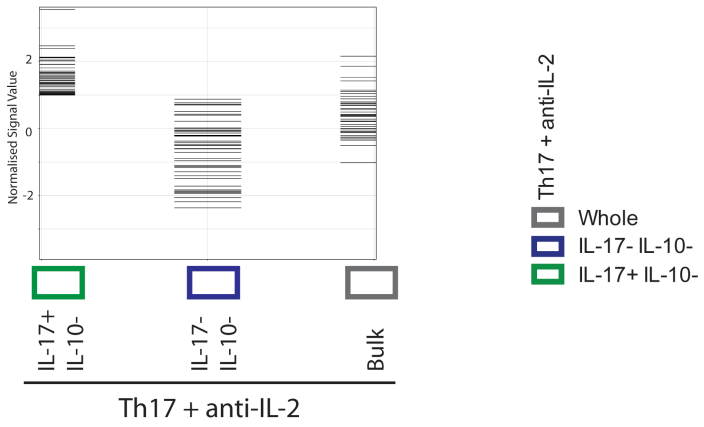


Figure 6B.12 Group 1: 58 genes decreased in the IL-17+ IL-10- subpopulations of Th17 cells cultured in the absence of IL-2

A. Genes in Group 1 from hierarchical clustering in Figure 6B.13. **B.** Expression profile of group. **C.** GO terms ($p<0.05$) and IPA pathways ($p<0.01$) with significant overlap, and examples of genes within the group. **D.** The read number of selected genes in each subpopulation. **E.** IPA network analysis of direct and indirect interactions between genes. Solid lines indicate direct interactions, the dashed lines indicate indirect interactions. Genes in red are those discussed.


A.
Th17 + anti-IL-2: Group 2
Gm7030
Postn
Sparc
Cald1
Lyz2
Cntln
H2-Q1
Col5a2
Gm5901
Nos2
Bgn
Fbn1
4930599N23Rik
Fbln5
E130218I03Rik
Larp6
Col1a2
Fstl4
Sulf2
Lox
1500017E21Rik
Loxl2
Thbs2
Rsc1a1
LOC100503496
Mpeg1
B3galt2
Kcnk10
Ifng
Tmod1
Tgfbi
Mmp3
Eda2r
Slc12a1
Timd2
Batf3
Tnc
Timp3
Fstl1
Gm10390
Col12a1
Cd70
Fn1
Col5a3
Iigp1
Cdh17
Col3a1
Fbln2
4
0
-4
Bulk
IL-17+ IL-10-
IL-17- IL-10-
Th17 + anti-IL-2

B.



C.

Associated GO terms	Associated IPA Pathways	Genes Included
Protein-lysine 6-oxidase activity, Protein cross-linking	Antigen presentation pathways, Communication between innate & adaptive immune cells, iNOS signalling	*Batf3, Nos2, Tgfbi, Ifng*

D.

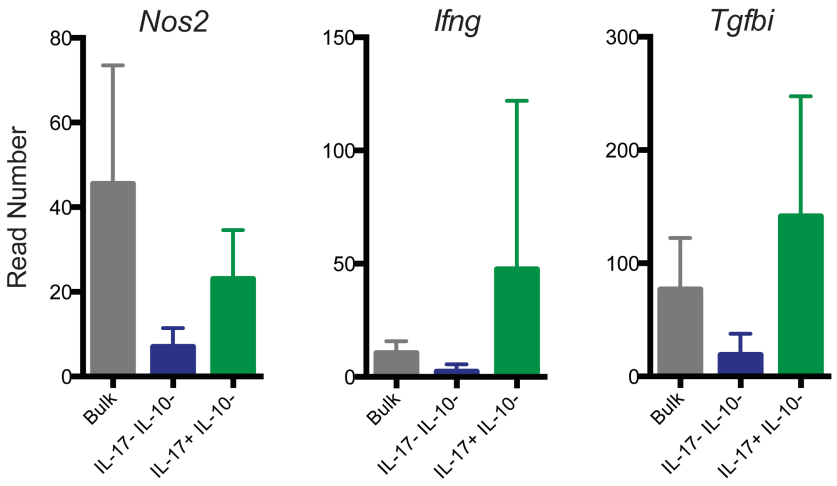


Figure 6B.13.1 Group 2: 48 genes increased in the IL-17+ IL-10- subpopulations of Th17 cells cultured in the absence of IL-2

A. Genes in Group 2 from hierarchical clustering in Figure 6B.13. **B.** Expression profile of group. **C.** GO terms ($p<0.01$) and IPA pathways ($p<0.01$) with significant overlap, and examples of genes within the group.

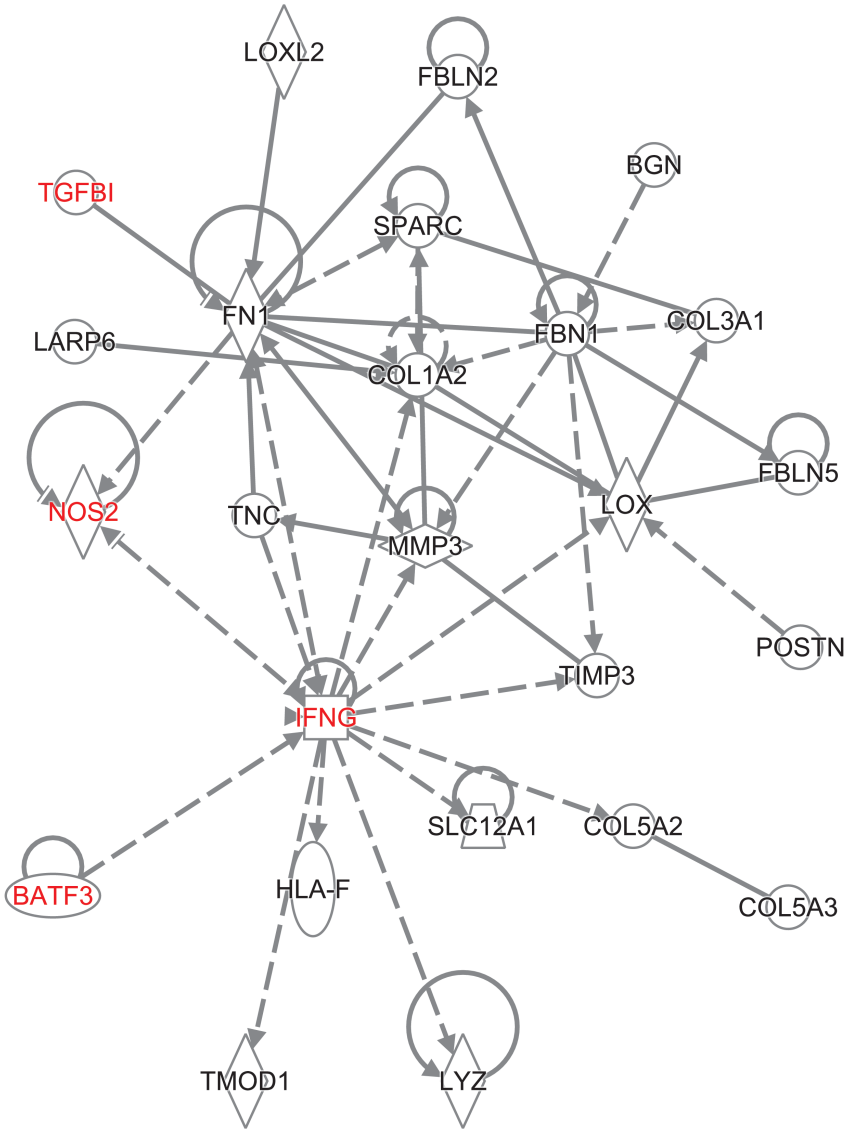
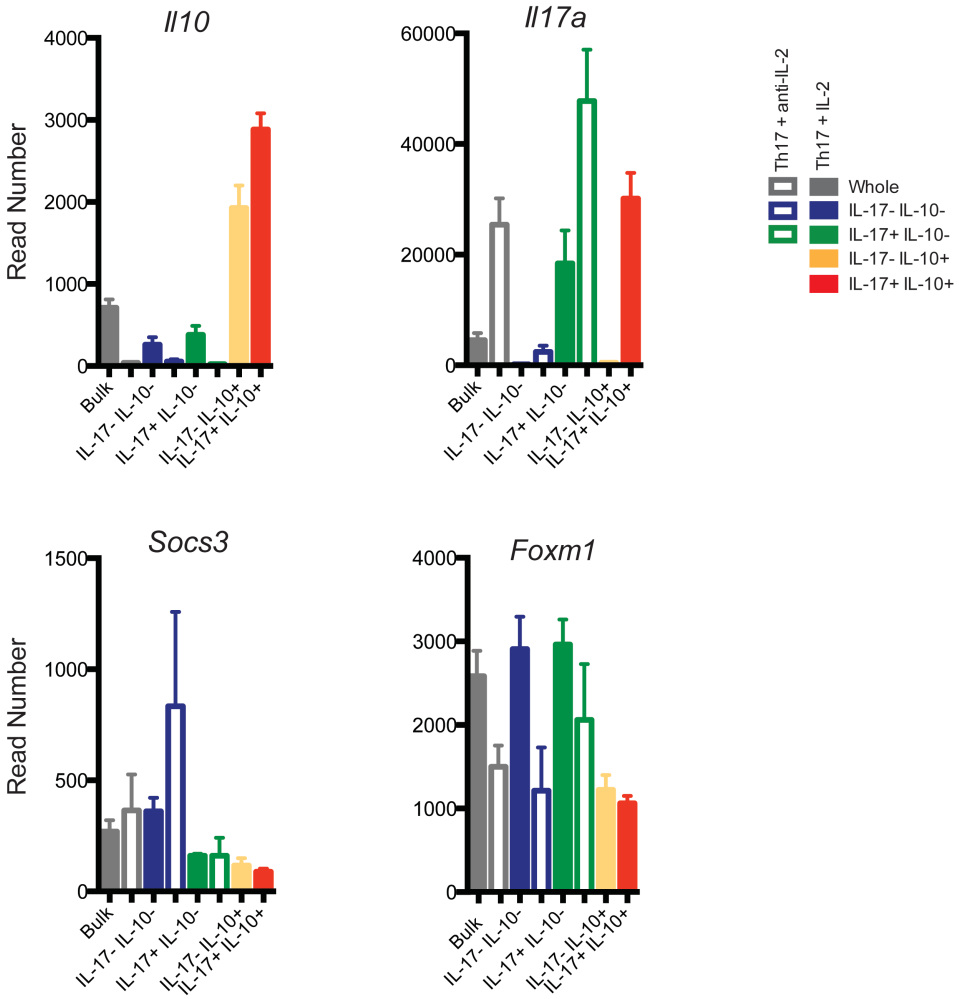


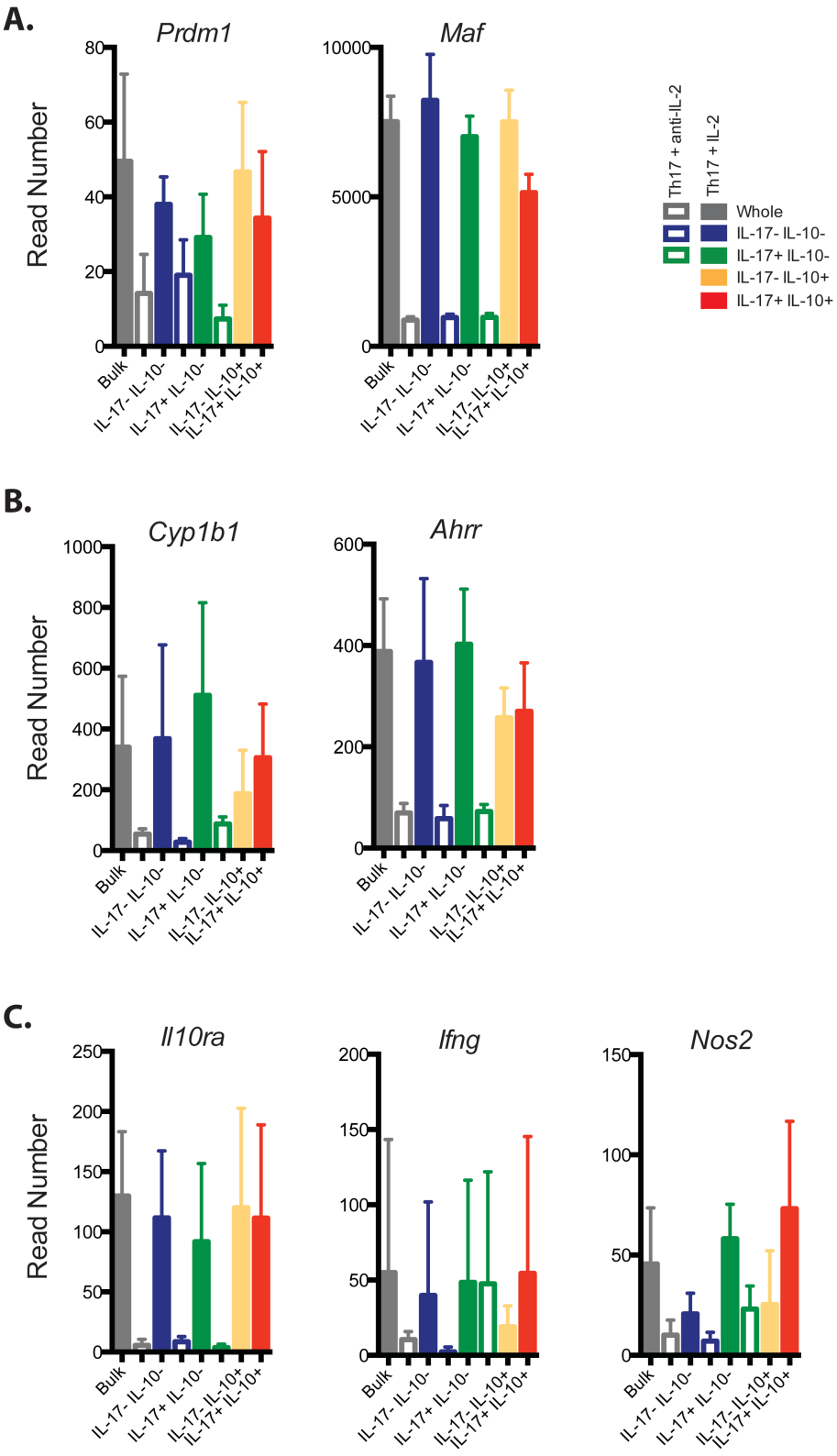
Figure 6B.13.2 Group 2: Network analysis of 48 genes increased in the IL-17+ IL-10- subpopulations of Th17 cells cultured in the absence of IL-2

IPA network analysis of direct and indirect interactions between genes. Solid lines indicate direct interactions, the dashed lines indicate indirect interactions. Genes in red are those discussed.



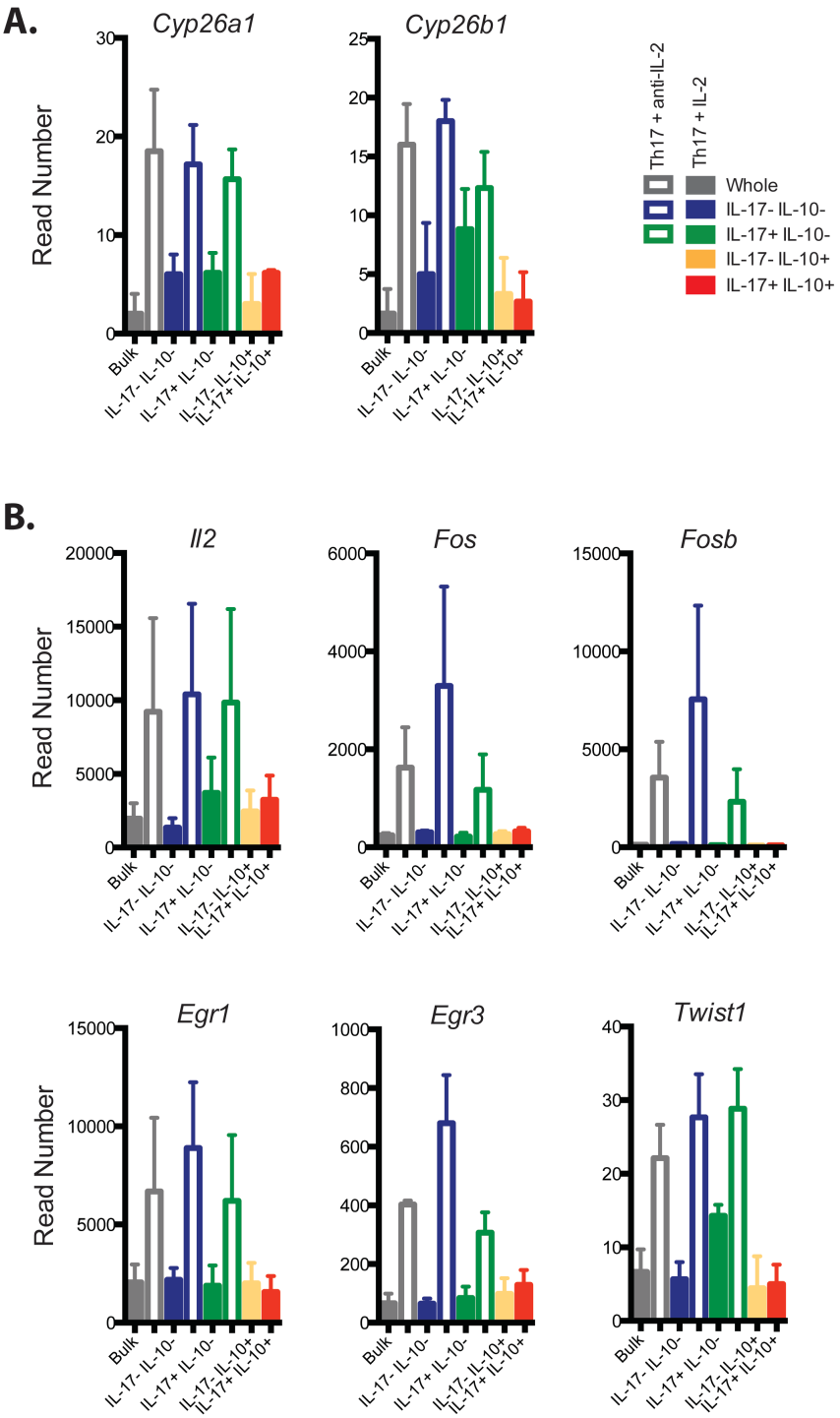
Discussion Figure 6.1 Genes of interest only revealed by analysis of the different intracellular cytokine producing subpopulations within the Th17 + IL-2 and Th17 + anti-IL-2 subsets

The read number of selected genes in each subpopulation.



Discussion Figure 6.2 Genes of interest upregulated by IL-2, revealed by analysis of the bulk unseparated Th17 + IL-2 and Th17 + anti-IL-2 subsets

The read number of selected genes in each subpopulation. **A.** Genes associated with discussion about Blimp-1 and c-Maf. **B.** Genes associated with discussion about AhR signalling. **C.** Other genes of interest upregulated by IL-2.



Discussion Figure 6.3 Genes of interest downregulated by IL-2, revealed by analysis of the bulk unseparated Th17 + IL-2 and Th17 + anti-IL-2 subsets

The read number of selected genes in each subpopulation. **A.** Genes associated with discussion about RA metabolism. **B.** Genes associated with ERK / AP-1 signalling.

Chapter 7. Summary and Future Perspectives

7.1 Summary

IL-10 has a vital role in maintaining a balanced and appropriate immune response (Jankovic et al., 2010; Saraiva and O'Garra, 2010). CD4+ T helper cells are important in regulating effective immune responses, and are a dominant source of IL-10 (Roers et al., 2004). However, owing to the complex nature of Th cell differentiation, a full understanding of the factors that regulate hallmark cytokine and IL-10 expression are incomplete. Thus, the conditions under which naïve $CD4^+$ T cells are polarised *in vitro* into different Th subsets have a fundamental impact on cytokine expression. The type of TCR stimulation, the cocktail and dose of polarising cytokines, the length of culture, and the duration of restimulation all play an important role in determining IL-10 and hallmark cytokine production (O'Garra et al., 2011). Therefore, in Chapter 3 we found the optimal *in vitro* differentiation conditions for Th1, Th2 and Th17 cell hallmark cytokine and IL-10 production, with regard to TCR stimuli, culture durations, restimulation kinetics, and cytokine stimuli.

The T helper cell subset populations are phenotypically heterogeneous, particularly with respect the IL-10 protein production. Each Th subset secretes a repertoire of different cytokines, but within that Th population the cells are heterogeneous – with cells having different cytokine secretion profiles. This confounds the ability to draw conclusions about the regulation of an individual cytokine within these heterogeneous populations as not all cells within that subset will express that cytokine. To better understand the common and disparate mechanisms involved in regulating *Il10* gene expression and hallmark cytokine gene expression, we wanted to separate the Th subsets into subpopulations and analyse the differential gene expression patterns within these populations using RNA-Seq. Therefore, in Chapter 4 we designed and optimised a system for extracting viable mRNA from intracellular cytokine stained samples. Using this protocol we separated different intracellular cytokine producing subpopulations within different Th1 and Th17 subsets and analysed their transcriptional profiles.

7.1.1 Major conclusions from studying the transcriptional profiles of Th1 and Th1 + IL-27 subsets, and Th17 + IL-2 and Th17 + anti-IL-2 subsets

Th1 cell differentiation is driven by IL-12, which alongside strong TCR ligation is required to initiate IL-10 expression in this cell type (Saraiva et al., 2009). Additionally, IL-27 has been shown to boost IL-10 expression in Th1 cells (Stumhofer and Hunter, 2008). Therefore in Chapter 5 we cultured Th1 cells in the presence or absence of IL-27 to see how this cytokine differentially affected gene expression in these cells. Of note, the *Ifng* and *Il10* mRNA expression profiles matched the IFNγ and IL-10 protein production profiles of these cells.

Th17 cells, primarily driven by TGFβ and IL-6, express IL-10 transiently and heterogeneously, via mechanisms that are still not understood. IL-23 has been shown to suppress IL-10 expression (Ghoreschi et al., 2010; McGeachy et al., 2007) and promote a 'pathogenic' phenotype of Th17 cells that produce proinflammatory cytokines including IFNγ. IL-2 with high levels of TGFβ is thought to be essential for Treg differentiation *in vitro* (Davidson et al., 2007; Yamane and Paul, 2012). We have found that IL-2 levels are crucial in driving two distinct forms of Th17 cell; addition of IL-2 results in increased IL-10, albeit alongside decreased IL-17, and blockade of IL-2 results in the disappearance of IL-10 alongside increased IL-17. Therefore in Chapter 6 we cultured Th17 cells in the presence of IL-2 or presence of anti-IL-2 to see how this cytokine differentially affected gene expression in these cells. Of note, the *Il17a* and *Il10* mRNA expression profiles matched the IL-17 and IL-10 protein production profiles of these cells.

Our results highlight that the Th1 and Th1 + IL-27 subsets are not only heterogeneous in cytokine production but also at the level of gene expression. Overall the analysis of the bulk unseparated Th1 and Th1 + IL-27 subsets exposed little about the effect of IL-27 on the transcriptional profiles of Th1 cells and it was hard to elucidate any mechanisms that may regulate *Il10* gene expression. By separating the different intracellular cytokine producing subpopulations we have gained a much greater understanding of the transcriptional changes that occur in Th1 cells in the presence or absence of IL-27, and of the transcriptional changes that occur upon cytokine production in Th1 and Th1 + IL-27 cells.

Analysis of the transcriptional profiles of the bulk unseparated Th17 + IL-2 and Th17 + anti-IL-2 subsets, revealed that the cells within each subset were transcriptionally very different. Unlike what we saw with the analysis of the Th1 and Th1 + IL-27 cells, analysis of the different intracellular cytokine producing subpopulations within the Th17 + IL-2 and Th17 + anti-IL-2 subsets revealed very similar results to the analysis of the bulk unseparated subsets. This is not in keeping with suggestions that Th17 cell populations are highly heterogeneous and that only by analysis at the single cell level can regulatory networks be established (Kuchroo VK, International Congress of Immunology, 2013). However, this may be explained by the IL-2 or anti-IL-2 conditions in this study resulting in considerably more homogeneous Th17 cell populations with very distinct transcriptional profiles.

This project has revealed that IL-27 predominantly results in the downregulation of gene expression in Th1 cells. The expression of certain glutamate receptors, which have been proposed to be involved in the inhibition of IL-10 production (Pacheco et al., 2006), was downregulated by IL-27. Furthermore, in this study IL-27 downregulated genes involved in metabolic processes, that are known to suppress the transcription of inflammatory responses (Cunard et al., 2004; Sakiani et al., 2013). Finally, our data shows that IL-27 appears to downregulate genes associated in AhR signalling in IFNγ+ IL-10- and IFNγ+ IL-10+ subpopulations.

We found that the expression of *Maf* was significantly upregulated by IL-27 in Th1 cells and IL-2 in Th17 cells (*Maf* expression was abolished when IL-2 signalling was blocked by anti-IL-2). We have shown that IL-27 (in Th1 cells) and IL-2 (in Th17 cells) drive IL-10 production in these cells, and therefore c-Maf could be a common factor used by IL-27 and IL-2 to drive *Il10* expression. Therefore, as alluded to in the literature, c-Maf seems to be associated with IL-10 production in Th cells, although the role of c-Maf in different Th subsets and the production of proinflammatory cytokines is unclear. c-Maf has been shown to have a role in the differentiation of many different Th subsets (Th1 (Neumann et al., 2014; Saraiva et al., 2009)) (Th2 (Hodge et al., 1996; Li et al., 1999a; Rengarajan et al., 2002)) (Th17 (Ciofani et al., 2012; Li et al., 2012a; O'Shea et al., 2011)) and therefore the mechanism by which it may regulate IL-10 (Apetoh et al., 2010; Kim et al., 1999; Neumann et al., 2014; Saraiva et al., 2009; Xu et al., 2009) is complex and remains unresolved. A comprehensive analysis of the role of

c-Maf in the differentiation and production of IL-10 in various Th cell subsets would be required to resolve the mechanisms by which this transcription factor regulates immune responses.

Additionally, we found that multiple molecules involved in AhR signalling were increased by IL-27 in Th1 cells and by IL-2 in Th17 cells, suggesting a role for this pathway in regulating IL-10 production. However, the AhR signalling molecules that were upregulated by IL-27 in Th1 cells were different from the molecules upregulated by IL-2 in Th17 cells. IL-27, in Th1 cells, resulted in the upregulation of *Arnt2*. IL-2, in Th17 cells, resulted in the upregulation of *Cyp1a1* and *Ahrr*. Therefore, our data suggests that though the AhR signalling pathway may have a role in regulating *Il10* expression in Th cells, it is highly complex.

7.2 Future Perspectives

IL-27 upregulates *Il10* expression in Th1 cells and we have shown that IL-2 upregulates *Il10* expression in Th17 cells. This study has revealed several factors that are upregulated by IL-27 in Th1 cells and are dependent on IL-2 in Th17 cells, such as *Maf* or *Prdm1*. Therefore these factors are possible targets that may be associated with the regulation of *Il10* gene expression. To ascertain the role of these factors in the regulation of *Il10* gene expression the O'Garra laboratory will utilise CRISPR-cas to knock out these genes *in vitro* and *in vivo*. Furthermore, using retroviral transfection of Th17 cells cultured in the presence of anti-IL-2 with these target genes, we could ascertain the role of these genes in the regulation of *Il10* gene expression; to see if reconstitution can result in the induction of *Il10* expression in Th17 + anti-IL-2 cells.

In particular we have revealed that the expression of *Maf* was significantly upregulated by IL-27 in Th1 cells and IL-2 in Th17 cells By using c-Maf deficient mice we can further address the role of c-Maf in Th cell differentiation and IL-10 production. Different Th subsets can be cultured *in vitro* to establish a system in which c-Maf may affect IL-10 production. Furthermore, *in vivo* infection models, which drive specific Th cell immune responses, can be assessed to determine the role of c-Maf in CD4+ T cell differentiation and IL-10 production. For instance, Malaria or Toxoplasma gondii can be used to drive Th1 responses, Helminth infection or allergy models effectively result

in Th2 responses, and fungal infection results in the differentiation of Th17 cells. Alongside phenotypic analysis of these animals during infection, cells can also be extracted for analysis *ex vivo*. Moreover, using the techniques optimised in this thesis, cells from these infections can be extracted and separated based on their intracellular cytokine profiles for further analysis by RNA- and ChIP-Seq. This would enable a comprehensive and in depth analyses of the role of c-Maf in Th cell differentiation and cytokine production, to resolve the mechanisms this transcription factor regulates in immune responses.

In the presence of anti-IL-2 many genes were increased in Th17 cells, alongside *Il17a*. Therefore these genes may be targets involved in inflammatory pathways within Th17 cells that drive pathogenesis. Again, to ascertain the role of these factors in the regulation of the expression of inflammatory factors the O'Garra laboratory will utilise CRISPR-cas to knock out these genes *in vitro* and *in vivo.*

7.3 Conclusion

In this thesis we have devised and optimised a technique that has never been applied to immune cells and will be greatly beneficial within the field for both *in vitro* and *in vivo* studies. We have shown that it is possible to extract high quality RNA from *in vitro* differentiated Th1 cells cultured in the presence or absence of IL-27 and Th17 cells cultured with IL-2 or anti-IL-2, even though they are fixed and stained for intracellular cytokines. By separating the different intracellular cytokine producing subpopulations from these different Th cell subsets according to their hallmark cytokine and IL-10 production we have revealed possible mechanisms by which IL-27 alters Th1 cells and IL-2 alters Th17 cells, and we have elucidated factors and pathways that may be involved in the regulation of *Il10* expression.

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